THE RESPONSE OF TREE LEGUMES TO INOCULATION WITH RHIZOBIA IN RELATION TO THEIR RHIZOBIAL SPECIFICITY AND THE DENSITY OF INDIGENOUS RHIZOBIA

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For my parents

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Chapter 1

Thesis Introduction

Nitrogen is the most limiting plant nutrient in agriculture (Singer and Munns, 1987). Despite its abundance, plants are unable to use N_2 directly. Most legumes, through symbiosis with rhizobia (in this thesis, rhizobia will be used to refer to both *Rhizobium* and *Bradyrhizobium*, unless specified differently), have the ability to reduce N_2 through biological nitrogen fixation (BNF) into a form usable for growth. Increasing demand for agricultural products combined with a need to conserve the world's limited resources make increased use of BNF in agriculture and forestry an important global objective. Fast-growing N_2 fixing leguminous trees are being widely promoted as sources of renewable energy and biologically fixed N for associated crops (Brewbaker et al., 1982; Dommergues 1987; Kang et al., 1984).

When appropriate rhizobia are absent from a soil, legume BNF does not occur. Then, inoculation of legumes with rhizobia can supply sufficient appropriate rhizobia to satisfy a plant's rhizobial requirements for maximal N_2 fixation and provide considerable return on investment in inoculant technology. However, where inoculant or needed infrastructure are not available, the cost of inoculant can be prohibitive. For farmers to determine if

inoculation is a wise investment and for regional planners to decide if and where to develop needed associated infrastructure, it is important for both to be able to predict whether or not a response to inoculation will occur and the magnitude of the response.

Several approaches have been advanced for assessing the need for inoculation. Field experiments have the advantage of directly measuring the effect of inoculation on yield. However, results from field experiments are site specific and their cost generally precludes wide use.

Another approach, the focus of this thesis, is to use factors that determine response to inoculation as indicators of the magnitude of the response. Relationships between these factors and response to inoculation can be used to predict responses to inoculation in other areas. Experiments with grain and forage legumes have identified density of indigenous rhizobia as the major determinant of response to inoculation (Singleton and Tavares, 1986; Thies et al., 1991a and 1991b). Available soil N also influences the magnitude of response to inoculation (Thies et al., 1991b).

The use of rhizobial density for assessing the need to inoculate involves three key components: (i) use of the most-probable-number plant-infection (MPN) assay, (ii) a knowledge of rhizobial specificity, and (iii)

establishment of the relationship between rhizobial density and response to inoculation. Because the accuracy of the MPN assay has not been established using trees and little is known about the rhizobial specificity of important tree legumes, these components will be addressed in Chapters 2 and 3 respectively. Chapter 4 examines the relationship between rhizobial density and response to inoculation of six tree species and the effect of available soil N on this relationship.

Chapter 2

Comparison of Most-Probable-Number Estimates of Tree Rhizobia with Plate Counts

ABSTRACT

Enumeration of rhizobia by most probable number plant infection (MPN) assays has been conducted with only a few leguminous tree species as hosts. To see if reasonable agreement could be obtained using conventional plant infection growth systems, MPN estimates were compared with plate counts of pure cultures using seven tree species grown in both growth pouches and on agar slants in glass tubes and seven other species grown in growth pouches alone. Reasonable agreement can be obtained between plate counts and MPN estimates, with closer agreement in agar slants than growth pouches for small-seeded species. Agar slants and growth pouches should be scored for nodulation 5 and 7 weeks after inoculation, respectively.

INTRODUCTION

Although there has been significant interest in the potential of fast-growing nitrogen-fixing leguminous trees as fuelwood, fodder, construction material, and a source of biologically-fixed N_2 for associated crops in agroforestry systems, there has been little systematic study of their associated rhizobia. The most probable

number plant-infection (MPN) assay, a technique widely used to enumerate populations of indigenous rhizobia, has only rarely been used with leguminous trees (Table 2.1). Rarer still are evaluations of its accuracy when used with trees. The purpose of this paper is to evaluate the accuracy of MPN assays with a variety of tree legumes grown in traditional growth systems through comparisons of plate counts and MPN estimates of the same pure rhizobial cultures.

The MPN assay is based on the capacity of rhizobia to form nodules on appropriate host plants under suitable conditions. MPN assays use the pattern of nodulated and non-nodulated plants inoculated with a dilution series to estimate the density of the rhizobial population in the original solution. Because no selective medium has proven reliable for distinguishing rhizobia from other microorganisms, the MPN assay is widely used for enumerating rhizobia in the presence of other microorganisms (Brockwell, 1980). Enumeration of indigenous rhizobia is especially useful for predicting the need to inoculate legumes with rhizobia (Brockwell et al., 1988; Thies et al., 1991b).

The agreement of plate counts with MPN estimates of pure rhizobial cultures provides a measure of compliance of the MPN assay with the underlying assumptions (see Scott and Porter, 1986) and a means for comparing the

appropriateness of different growth systems for different legume species. An important assumption is that a single rhizobial cell causes nodule formation (Cochran, 1950). Although agreement between plate counts and MPN assays with pure cultures does not guarantee accuracy in the presence of other microorganisms, it is an essential step for establishing the reliability of the technique. While reasonable agreement has been obtained in some cases with grain and forage legumes, there is a tendency of MPN assays to underestimate plate counts (Scott and Porter, 1986). Boonkerd and Weaver (1982) reported underestimations greater than 100 fold.

It is important to keep in mind that plate count-MPN comparisons cannot be evaluated using conventional statistical approaches. The fiducial limits developed by Cochran (1950) were based solely on the dilution ratio and the number of replicate growth units per dilution level, given the assumptions of the assay. When the assumptions are not met, the actual fiducial limits will be higher than those calculated from theory (Scott and Porter, 1986).

A variety of growth systems have been used in MPN assays, including agar slants, tubes and cups filled with sand or horticultural vermiculite, Leonard jar assemblies (Leonard, 1944), and plastic growth pouches (Gibson, 1980). Agar slants have been recommended for legumes

whose seeds are smaller than those of vetch (Vincent, 1970). Growth pouches are particularly useful for larger-seeded legumes.

No studies have investigated the number of weeks growth units need to be kept prior to scoring. Published reports of MPN assays conducted with tree legumes indicate that growth units have been kept for 3 to 12 weeks after inoculation before scoring (Table 2.1). It is unclear from these reports whether 3 or 4 weeks was sufficient for nodulation to occur or whether longer periods were essential.

The objectives of the experiments presented in this paper were to (i) evaluate the accuracy of the MPN assay for a variety of tree legumes; (ii) compare the suitability of growth pouches and agar slants for seven tree legumes; and (iii) determine how long the assay should continue.

MATERIALS AND METHODS

Growth systems

Two growth systems were used in this study. *Acacia auriculiformis*, *Acacia mangium*, *Acacia mearnsii*, *Leucaena diversifolia*, *Paraserianthes falcataria*, *Robinia pseudoacacia*, and *Sesbania grandiflora* were grown in plastic growth pouches (Northrup King Co.) and on agar slants in 25 mm x 250 mm glass tubes. Additional MPN

assays were conducted in pouches with *Albizia lebbeck*, *Albizia saman*, *Calliandra calothyrsus*, *Enterolobium cyclocarpum*, *Flemingia macrophylla*, *Gliricidia sepium*, *Leucaena leucocephala*, and *Sesbania sesban*.

Agar slants were prepared according to Somasegaran and Hoben (1985) using 15 ml per tube of a plant nutrient solution containing 0.482 mM P, 0.96 mM K, 0.50 mM S, 0.21 mM Mg, and 0.29 mM Ca as K_2HPO_4, $MgSO_4 \cdot 7H_2O$, and $CaSO_4 \cdot 2H_2O$; 10 μmFe as FeEDTA; 0.0625 ml l^{-1} of a micronutrient solution (Liquid Hawaiian Horticultural Mix, Monterey Chemical Co.); and 15 g agar l^{-1}. After autoclaving, the agar was slanted at an angle of 10° from the horizontal, producing a slant approximately 10 cm long, beginning at the base of the tube. The slant was designed to promote maximal contact between roots and rhizobia at the base of the slant and at the bottom of the tube. Growth pouches were prepared by filling each pouch with 50 ml of the same solution used in the tubes, minus the agar. An additional 50 ml of the same solution was added prior to inoculation. After inoculation pouches were provided with sterile water as needed until harvest.

Plant Culture and Selection

Seeds were scarified and surface sterilized with appropriate treatments (Table 2.2). After allowing the seeds to imbibe water for 5-12 hours, they were germinated on water-agar in petri plates according to Somasegaran and

Hoben (1985). Two to 5 days later, when the radicles had reached 0.5 to 1.5 cm in length, the seedcoats of uniform seedlings were carefully removed to prevent hardening of the seed coat and to facilitate examination of tubes for nodulation. In tubes, seedlings were placed on the surface of the agar, with the root collar approximately 6 to 7 cm from the bottom of the slant. One and two seedlings were planted in tubes and pouches respectively. After planting, racks of pouches and tubes were placed in a growth room receiving > 300 μEinsteins m^{-2} s^{-1} of photosynthetically active radiation at plant height from 1000 W high pressure sodium lamps for 16 hours day^{-1}.

Prior to inoculation growth units with poorly growing plants were eliminated, leaving enough uniform plant growth units for at least 6 dilution levels with 4 replicate growth units each. In tubes, seedlings with tap roots that penetrated the agar were eliminated in accordance with the observation of Woomer et al. (1988b) that penetration of the agar by *L. leucocephala* roots resulted in poor nodulation. Of the trees grown in tubes for these experiments, *A. mangium* roots had the greatest tendency to penetrate the agar.

Inoculation

Rhizobial strains (Table 2.3) were grown for 6-10 days in either yeast-extract mannitol broth (Vincent, 1970) or arabinose-gluconate medium (Sadowsky et al.,

1987). Prior to inoculation, cultures were diluted in a solution containing the salts found in yeast-extract mannitol broth (Vincent, 1970) with 0.01 % Tween 80 (Fisher Scientific Co.) added as a surfactant. Dilution procedures followed those outlined in Somasegaran and Hoben (1985). Either four-fold or 10-fold dilutions were used.

Seedlings were inoculated 7 to 12 days after planting, when root radicles reached the bottom of the slants or had begun to differentiate into secondary roots in pouches. One ml of diluted rhizobial culture was applied directly to the roots in pouches and to the lower portion of the roots in tubes. An average of seven uninoculated growth units were included per MPN assay as checks for contamination within the system. Uninoculated controls received 1 ml of rhizobia-free diluent.

Plate Counts

Plate counts were conducted at the time of inoculation using the Miles and Misra drop plate method as modified by Somasegaran and Hoben (1985). At least four replicate 0.03 ml aliquots were used from each of three dilution levels from the inoculation dilution series. Prior to data collection, plates were incubated at 27°C for 7 to 10 days for slow-growing strains, and 3 to 5 days for fast-growing strains.

MPN determinations

With two exceptions scored at 4 weeks, growth units of MPN assays were scored for nodulation 5 to 7 weeks after inoculation. The most probable number was determined using a computer program (Woomer et al., 1990), beginning with the highest dilution step in which all growth units nodulated.

Assessment of time to scoring

MPN assays in pouches and tubes of *A. auriculiformis*, *A. mangium*, *A. mearnsii*, and *R. pseudoacacia*, were scored weekly for nodulation from the second to the seventh week after inoculation. Weekly scores were also recorded for *S. grandiflora* and *L. diversifolia*, grown in pouches and tubes respectively.

RESULTS

The plate count was within the corresponding 95% confidence interval of the MPN (Cochran, 1950) for 18 of 54 plate count-MPN comparisons (Appendix B summarized in Table 2.4), indicating lack of a significant difference between them. However, in all but three plate count-MPN comparisons the MPN underestimated the plate count. The rhizobial density of the original solution ranged from 3.67×10^7 to 7.55×10^9 rhizobia ml^{-1} as measured by the plate count. Over all MPN assays, only five of 384

uninoculated controls nodulated, with never more than one nodulated control per assay.

In tubes, all species with seed weights less than 25 mg seed^{-1} (Table 2.1) had average plate count:MPN estimate (PC:MPN) ratios of less than 26. *A. mearnsii*, *L. diversifolia*, and *P. falcataria* in tubes and *L. diversifolia*, *C. calothyrsus*, and *S. sesban* in pouches all had average PC:MPN ratios less or equal to 2.1. *S. grandiflora*, with a seed weight of 36 mg/seed, had an average PC:MPN ratio of 1428.9 in tubes but only 5.8 in pouches. In pouches, seven of 14 species had average PC:MPN ratios of less than 35. For 11 of 14 tree species, PC:MPN ratios of less than 35 were obtained in either tubes or pouches.

Five of 10 MPN estimates from nodulation scores taken weekly from 2 to 7 weeks after inoculation increased after the fourth week (Figure 2.1). None of the 48 uninoculated controls nodulated in these assays. For species grown in both pouches and tubes, the average number of weeks to arrive at the final MPN estimate were 6.0 in tubes compared with 4.8 in pouches. In tubes, the MPN estimates of *A. auriculiformis* and *A. mearnsii* increased during the interval from 6 to 7 weeks after inoculation whereas in pouches, the MPN estimate of only one species, *A. mangium*, increased in the interval from 5 to 6 weeks and none after 6 weeks.

DISCUSSION

The excellent agreement between plate counts and MPN estimates observed with *A. mearnsii*, *L. diversifolia*, and *P. falcataria* in tubes and with *L. diversifolia*, *C. calothyrsus*, and *S. sesban* in pouches, indicates that relatively accurate measurement of rhizobial density can be achieved with pure cultures for a range of tree legumes. Tree legumes appear to be no different from herbaceous legumes in this respect. A high level of agreement between MPN estimates and plate counts (PC:MPN ratio < 6) has been obtained with many herbaceous legumes including clover (Brockwell, 1963; Tuzimura and Watanabe, 1961), alfalfa (Weaver and Frederick, 1972; Scott and Porter, 1986), chick pea (Toomsan et al., 1984), and soybean (Weaver and Frederick, 1972; Brockwell et al., 1975).

Growth pouches were not suitable for seven of 14 tree species and tubes not suitable for *S. grandiflora*. These species had PC:MPN ratios > 100, indicating that more than 100 rhizobia were required for nodule formation. Other authors have reported large discrepancies between plate counts and MPN estimates. Boonkerd and Weaver (1982) reported consistent underestimations involving *Bradyrhizobium* strains applied to cowpea and siratro. They obtained PC:MPN ratios of 260.6 for cowpea in pouches (mean of 4 strains), 255.0 for siratro in pouches (mean of

five strains), 125.6 for siratro in vermiculite-filled cups (mean of two strains), and 15.3 for siratro in agar slants (mean of two strains). In our assays, of seven species grown in both pouches and tubes, good agreement of five species in only one of two growth systems suggests that the problems in obtaining agreement are growth-system related, rather than indicative of an inherent inability of trees to nodulate with a single rhizobial cell, as was suggested by Boonkerd and Weaver (1982) for cowpea and siratro.

Our results demonstrate the appropriateness of agar slants for small-seeded tree species and emphasize the need to tailor the growth system to the needs of the plant. Vincent (1970) reported that enclosed tubes were only appropriate for legumes with seed weight less than that of "vetch" (approximately 25 mg seed^{-1}). *S. grandiflora*, with a seed weight of 36.4 mg seed^{-1}, was the only species tried that did not grow well in tubes. Woomer et al. (1988b) noted the importance of placing the diluted inoculant directly on the roots of the plants to get good nodulation and noticed that the roots of *Macroptilium atropurpureum* tended to accumulate at the bottom of the tube without growing extensively on the surface of the slant where the inoculant was applied. Observations in our experiments confirmed that root growth of the *Acacia* spp. in particular was not extensive,

especially in tubes, with most nodulation occurring at the bottom of the tubes. Therefore, the quantity of agar we used and the angle of the slant were designed to channel both roots and rhizobia directly to the bottom of the tube to ensure maximal contact between them. We found that 15 ml of agar was sufficient to support growth of the tree species we used for the duration of the assays.

Weekly assessment of MPN estimates (Figure 2.1) indicates the importance of keeping pouches for at least 5 weeks and tubes for at least 7 weeks after inoculation. Two to 3 weeks had been recommended as adequate by Vincent (1970), and 4 weeks by Brockwell (1980), based on MPN assays with herbaceous legumes. Scoring at 3 weeks had also been recommended as adequate for cowpea and siratro in pouches (Boonkerd and Weaver, 1982).

Reports of times to assessment of MPN assays with tree legumes (Table 2.1) indicate a range from 2-3 weeks to 11 weeks after inoculation, with nodulation in four of seven reports assessed at 3 weeks or less. The agreement Davis (1982) obtained between an MPN estimate and plate counts with *L. leucocephala* (PC:MPN ratio < 1) was from plants scored for nodulation approximately 11 weeks after inoculation.

In conclusion, our results indicate that reasonable agreement between plate counts and pure rhizobial cultures can be obtained with tree legumes but that results are

very species and growth system dependent. MPN estimates using agar slants agreed well with plate counts for *A. auriculiformis*, *A. mangium*, *A. mearnsii*, *L. diversifolia*, *P. falcataria*, and *R. pseudoacacia*, while growth pouches gave acceptable agreement for *C. calothyrsus*, *G. sepium*, *L. diversifolia*, *L. leucocephala*, *R. pseudoacacia*, *S. grandiflora*, and *S. sesban*. Because of delayed nodulation, it is recommended that tubes and pouches be scored for nodulation at least 5 and 7 weeks after inoculation respectively. These results provide a basis for selecting appropriate growth systems for MPN assays with tree legumes and suggest that accurate MPN assays can be obtained in soils.

Table 2.1

MPN assays conducted with tree legumes.

Reference	Species	Growth System	Time to Assessment[a]
Davis, 1982	*Leucaena leucocephala*	agar deeps	up to 11 weeks
Sanginga et al., 1985	*L. leucocephala*	not recorded	2-3 weeks[b]
Sanginga et al., 1987	*L. leucocephala*	not recorded	2-3 weeks[b]
Singleton and Tavares, 1986	*L. leucocephala*	growth pouches	2-3 weeks
Singleton et al., 1991a	*L. leucocephala*	not recorded	not recorded
Thies et al., 1991a	*L. leucocephala*	growth pouches	3-4 weeks
Woomer et al., 1988a	*L. leucocephala*	agar slants	7 weeks
Woomer et al., 1988b	*L. leucocephala*	agar slants	2-3 weeks
Virginia et al., 1987	*Prosopis glandulosa*	dibble tubes	6 weeks
Singleton et al., 1991a	*Sesbania sesban*	not recorded	not recorded

[a] weeks after inoculation
[b] according to the reference cited, Vincent (1970).

Table 2.2. Seed sources, weight, and scarification and surface sterilization methods.

Species	Seed Source[a]	Weight mg seed^{-1}	Scarification[b]	Surface Sterilization[c]
A. auriculiformis	AOP	24.0	A (15 min) +B	
A. mangium		9.8	A (20 min) +B	
A. mearnsii	local	16.8	A (20 min) +B	
A. lebbeck	AOP	135.2	C	D (1 min)
A. saman	AOP	221.2	C	D (1 min)
C. calothyrsus	NFTA	51.6	none	D (1 min)
F. macrophylla	IITA	17.2	C	D (1 min)
G. sepium	IITA	131.2	none	D (1 min)
L. diversifolia	local	18.4	A (15 min)	
L. leucocephala K 8	NFTA	46.8	A (15 min)	
P. falcataria	Dr. I.L. Domingo, Phillipines	21.6	A (20 min)	
R. pseudoacacia	Pakistan Forest Inst.	17.8	A (20 min) +B	
S. grandiflora	AOP	36.4	C	D (2 min)
S. sesban	NFTA	14.6	C	D (1-2 min)

[a]AOP, Agroforestry Outreach Project, Port au Prince, Haiti; IITA, International Institute of Tropical Agriculture, Ibadan, Nigeria; local, Maui, Hawaii; NFTA, Nitrogen Fixing Tree Association, Waimanalo, Hawaii.

[b]A = soaked in concentrated sulfuric acid for no. of minutes in parentheses, followed by 8-10 rinses with sterile water.

B = seeds covered with boiling water following the last rinse in treatment A and allowed to cool.

C = seeds scarified mechanically, either nicked with scissors or a scalpel, in one case (*Sesbania sesban*), scarified by scratching with sand paper.

[c]D = seeds soaked in 50 % bleach for the no. of minutes in parentheses, followed by 8-10 rinses in sterile water.

Table 2.3

Strains used in MPN assays.

Host Species in MPN Assay	Strain	Rhizobial genus[a]	Original Host	Other Name
Acacia auriculiformis	TAL 569	*Bradyrhizobium*	*Desmodium uncinatum*	MAR 472
Acacia auriculiformis	TAL 651	*Bradyrhizobium*	*Calopogonium mucunoides*	UMKL 44
Acacia auriculiformis	TAL 1446	*Bradyrhizobium*	*Acacia auriculiformis*	-
Acacia mangium	TAL 1867	*Bradyrhizobium*	*Acacia mangium*	LB 5
Acacia mearnsii	TAL 940	*Bradyrhizobium*	*Acacia mearnsii*	Num 777
Acacia mearnsii	TAL 941	*Bradyrhizobium*	*Acacia mearnsii*	Num 778
Acacia mearnsii	TAL 1388	*Bradyrhizobium*	*Acacia mearnsii*	-
Albizia lebbeck	TAL 1536	*Bradyrhizobium*	*Albizia lebbeck*	-
Albizia saman	TAL 833	*Bradyrhizobium*	*Albizia saman*	UMKL 27
Calliandra calothyrsus	TAL 1455	*Bradyrhizobium*	*Calliandra surinamensis*	-
Flemingia macrophylla	TAL 1883	*Bradyrhizobium*	*Flemingia macrophylla*	Nit 52A1
Gliricidia sepium	TAL 1806	*Rhizobium*	*Gliricidia sepium*	BR 8801
Gliricidia sepium	TAL 1145	*Rhizobium*	*Leucaena leucocephala*	CIAT 1967
Leucaena leucocephala	TAL 1145	*Rhizobium*	*Leucaena leucocephala*	CIAT 1967
Paraserianthes falcataria	TAL 45	*Bradyrhizobium*	*Paraserianthes falcataria*	-
Robinia pseudoacacia	TAL 1889	*Rhizobium*	*Robinia pseudoacacia*	USDA 3436
Sesbania grandiflora	TAL 1114	*Rhizobium*	*Sesbania sp.*	IC 71
Sesbania grandiflora	TAL 1119	*Rhizobium*	*Sesbania sp.*	IC 91
Sesbania sesban	TAL 674	*Rhizobium*	*Sesbania rostrata*	-
Sesbania sesban	TAL 1042	*Rhizobium*	*Sesbania longifolia*	Nit 145B1

[a] = determined by IPTG XGal assay in conjunction with growth on sucrose and lactose (Appendix A).

Table 2.4

Comparison of MPN Estimates with Plate Counts (PC) for Tree Legumes

Species	No. of MPN Assays Performed	No. with plate count within 95% C.I. of MPN	Average PC:MPN Ratio	Standard Deviation of PC:MPN Ratios	Range of PC:MPN Ratios
AGAR SLANTS					
Acacia auriculiformis	3	0	25.7	29.2	5.0 - 67.0
Acacia mangium	4	0	16.0	6.4	5.0 - 21.0
Acacia mearnsii	4	3	2.0	0.9	0.6 - 2.2
Leucaena diversifolia	4	2	1.6	1.6	0.3 - 4.2
Paraserianthes falcataria	2	2	1.4	0.4	1.0 - 1.9
Robinia pseudoacacia	3	1	14.7	19.9	0.2 - 42.9
Sesbania grandiflora	2	0	>1428.9	1252.7	176.2 - >2682
GROWTH POUCHES					
Acacia auriculiformis	3	0	8057.7	9305.3	746.4 - 21189.2
Acacia mangium	3	0	2370.3	2450.7	517.3 - 5833.3
Acacia mearnsii	3	0	234.8	300.2	5.5 - 658.9
Leucaena diversifolia	2	2	1.3	0.4	1.0 - 1.7
Paraserianthes falcataria	2	0	548.7	335.6	213.1 - 884.4
Robinia pseudoacacia	3	1	5.9	6.8	0.1 - 15.4
Sesbania grandiflora	3	1	5.8	2.9	1.9 - 8.8
Albizia lebbeck	1	0	118.8		
Albizia saman	1	0	12823.5		
Calliandra calothyrsus	1	1	2.1		
Flemingia macrophylla	1	0	865.1		
Gliricidia sepium	2	1	7.1	5.9	1.1 - 13.0
Leucaena leucocephala	3	1	34.3	26.8	0.7 - 66.3
Sesbania sesban	3	3	1.2	0.4	0.9 - 1.8

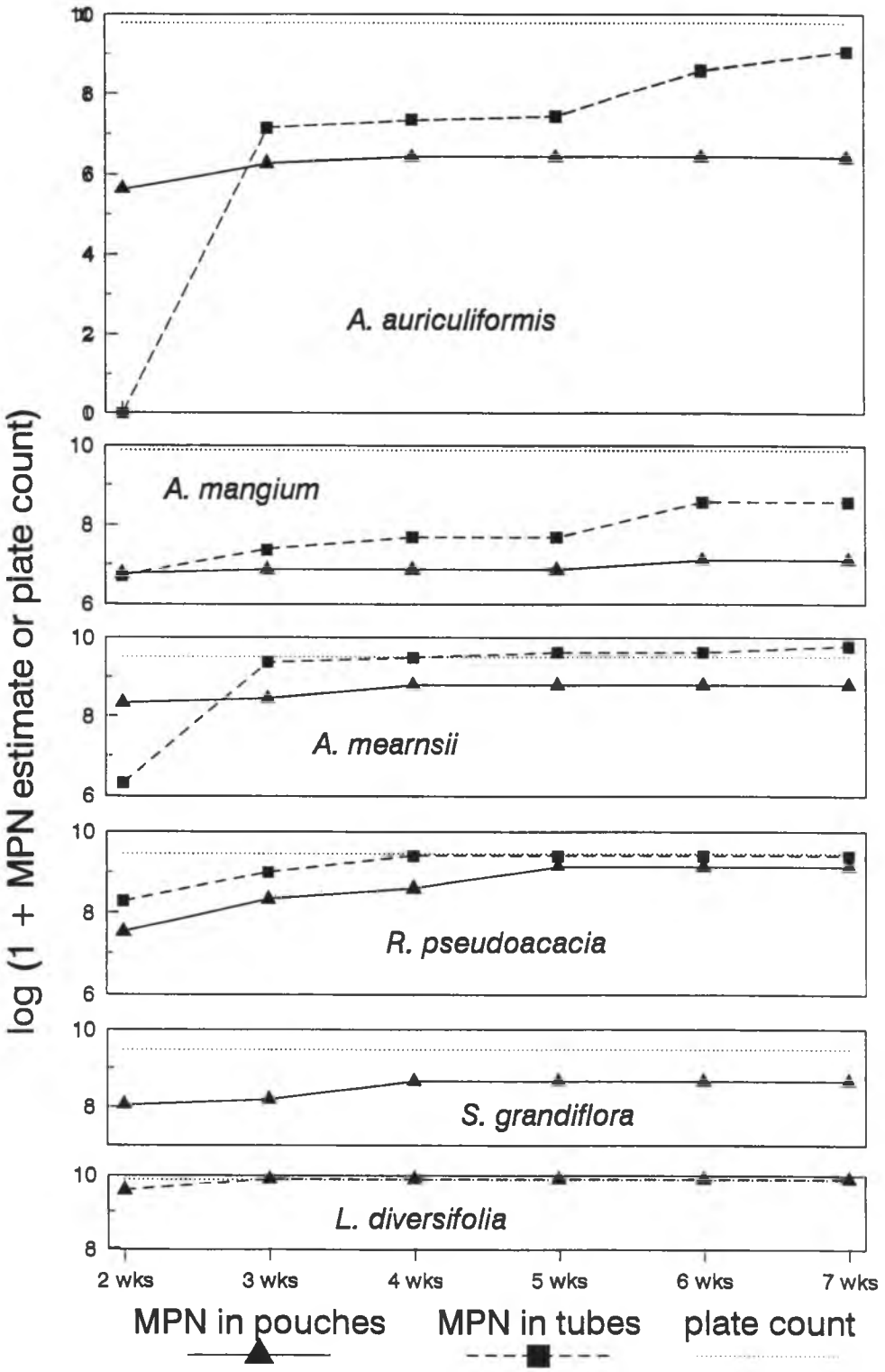


Figure 2.1. Weekly assessment of MPN estimates.

Chapter 3

Rhizobial Specificity of Fast-Growing Tree Legumes

ABSTRACT

Rhizobial specificity, defined in terms of nodulation and BNF effectiveness characteristics of a group of rhizobia on a host legume, has served as a basis for predicting the need to inoculate, selecting species for most-probable-number plant-infection assays, and preparing rhizobial inoculants suitable for a range of legume species. A series of cross-inoculation experiments were performed under growth room and greenhouse conditions to delineate rhizobial specificity of a variety of tree legumes. *Gliricidia sepium*, *Calliandra calothyrsus*, and *Leucaena leucocephala* nodulated effectively with rhizobia isolated from each of the three genera. With a few exceptions, *Sesbania grandiflora* and *Robinia pseudoacacia* nodulated effectively only with rhizobial strains isolated from each genus respectively. Considerable specificity was found among species that nodulate with *Bradyrhizobium*. While *Acacia mearnsii* nodulated with most strains but fixed N_2 effectively with relatively few, *Acacia mangium* and *Lysiloma latisiliqua* were specific for both nodulation and effectiveness.

INTRODUCTION

Nitrogen-fixing leguminous trees (NFLTs) are being planted on a wide scale in the tropics to provide fuelwood, construction materials, fodder, and nitrogen-rich biomass for improving soil fertility. Although biological nitrogen fixation is an important attribute of NFLTs, little has been done to delineate their rhizobial specificity in terms of nodulation and effectiveness.

Cross-inoculation is the reciprocal application of rhizobia isolated from different legumes to each other. A series of cross-inoculation experiments were conducted with a variety of tree legumes to delineate their rhizobial specificity in terms of nodulation and effectiveness.

Trees, in general, nodulate effectively and almost exclusively with either fast-growing rhizobia (genus *Rhizobium*) or slow growing-rhizobia (genus *Bradyrhizobium*) (Dreyfus et al., 1987). A few species nodulate effectively with some rhizobial strains from both genera such as *Acacia seyal*, *Acacia sieberana* (Dreyfus and Dommergues, 1987; Dreyfus et al., 1987), *Acacia longifolia* (Barnet et al., 1985), and *Prosopis glandulosa* (Jenkins et al., 1987). Tree species that nodulate effectively with fast-growing rhizobia are specific for nodulation and effectiveness (Duhoux and Dommergues, 1985; Gibson et al., 1982; Trinick, 1982) whereas species that nodulate with

Bradyrhizobium are less specific (more promiscuous) for both characteristics (Graham and Hubbell, 1975; Date, 1977 and 1982). Trees that nodulate with *Rhizobium* have not been examined extensively for specificity for effectiveness with respect to each other. Similarly, specificity for effectiveness has not been systematically evaluated in tree species that nodulate with *Bradyrhizobium*. There is evidence that some non-woody legume species and cultivars that nodulate with *Bradyrhizobium* are specific for effectiveness and/or nodulation (Date and Norris, 1979; Burton, 1952; Keyser and Cregan, 1987, Thies et al., 1991c) and that *A. mangium* is specific for effectiveness (Galiana et al., 1990).

There is considerable confusion surrounding the generic classification of rhizobia that effectively nodulate some tree species (Table 3.1). Apparent misidentification of the genus of rhizobia that effectively nodulates some trees (eg. *Calliandra* and *Gliricidia*) is misleading given the view that species that nodulate effectively with *Rhizobium* are more likely to respond to inoculation than species that nodulate effectively with *Bradyrhizobium* (Dommergues, 1987; Dreyfus and Dommergues, 1981; Peoples et al., 1989).

The use of a small-seeded legume has been recommended for performing most-probable-number plant-infection (MPN) assays to determine the rhizobial population density of

symbiotically related legumes (Vincent, 1970; Brockwell, 1980 and 1982). The only recommended substitution involving trees has been *Desmanthus virgatus* for *Leucaena leucocephala* (Brockwell, 1980 and 1982; Davis, 1982). *Macroptilium atropurpureum* has been recommended as a substitute for "cowpeas and symbiotically related species" (Brockwell, 1980 and 1982). Whether or not bradyrhizobial populations nodulating tree legumes can be enumerated accurately with *M. atropurpureum* has not been investigated.

Effectiveness groups of legumes are subsets of cross-inoculation groups that respond similarly to a set of rhizobial strains (Burton, 1979). They have served primarily as a guideline for inoculant preparation. Only four tree species have been ascribed to effectiveness groups: *Leucaena leucocephala*, *L. retusa*, *Robinia pseudoacacia*, and *Albizia julibrissin* (Burton, 1979; NiFTAL and FAO, 1984).

To delineate effectiveness groups of 14 tree legumes in terms of nodulation and effectiveness, two cross-inoculation experiments were performed in pouches. The purpose of the first experiment, termed Pouch Experiment A, was to evaluate fast-growing tree legumes using strains of both *Rhizobium* and *Bradyrhizobium*. The purpose of the second experiment, Pouch Experiment B, was to evaluate the specificity of a larger group of trees known to nodulate

effectively with strains of *Bradyrhizobium*, using a larger number of these strains.

To confirm that data from the pouch experiments were not artifacts of the pouch system, effectiveness experiments were performed in pots in a greenhouse with three species, *A. auriculiformis*, *A. mangium*, and *A. mearnsii*. An additional objective of the pot experiments was to evaluate the effectiveness of a group of homologous strains (strains tested on the same species from which they were isolated) with *A. auriculiformis* and *A. mearnsii*.

MATERIALS AND METHODS

Tree species and rhizobial strains

Tree species (Table 3.2) were selected for their importance in reforestation and agroforestry in the tropics. Cultivars and seed sources are the same as described in Chapter 2 except for *Lysiloma latisiliqua*, obtained from Agroforestry Outreach Project, Haiti; *Enterolobium cyclocarpum* obtained from trees on Maui, Hawaii; and *Vigna unguiculata* cv. knuckle purple hull. Pouch experiment R-B evaluated seven tree species inoculated with 35 rhizobial strains. In pouch experiment B eight tree species were used that were either known to nodulate with bradyrhizobial strains from written reports (Allen and Allen, 1981) or from experience at the NifTAL

project. *M. atropurpureum* and *V. unguiculata* were also included in Pouch Experiment B because they are recognized as nodulating effectively with a wide variety of bradyrhizobial strains (Vincent, 1970). Eight uninoculated pouches were included for each tree species.

Rhizobial strains (Table 3.3) were from the NiFTAL Project (1000 Holomua Ave., Paia, HI). As much as possible, in Pouch Experiment A at least three strains isolated from each tree species (or genus) were used. In Pouch Experiment B 34 *Bradyrhizobium* strains were used including NifTAL's recommended strains for cowpea (TAL 173, 209, 658), peanut (TAL 1000, 1371), and lima bean (TAL 22, 209, 658). The same set of strains was used on each species. Strains used in the pot experiments were a subset from Pouch Experiment B, including the best strains identified from each of the three *Acacia* species. In addition, all strains isolated from *A. mearnsii* and *A. auriculiformis* in the NiFTAL collection were used to inoculate their respective homologous hosts.

Growth systems and experimental design

Pouch experiments

In the pouch experiments, trees were grown in growth pouches (Northrup King Co.), with a single plant of each of two species grown in each pouch (Table 3.2). Pouches were prepared as described in Chapter 2. Growth room conditions were the same as used in Chapter 2.

Four replicates of each treatment were included in Pouch Experiment A, with six uninoculated controls per species. Six uninoculated controls were included per species in Pouch Experiment B. In Pouch Experiment A, pouches were not randomized but received uniform light. Other experiments in the growth room had not shown a significant differences across blocks (data not shown). For Pouch Experiment B, pouches were randomized in racks (Somasegaran and Hoben, 1985) in three blocks. Every two days racks were rotated around the table to ensure uniformity of light for all racks.

Pot experiments

In the pot experiments, plants were grown in 1 l plastic pots containing moistened horticultural vermiculite (Grace and Co.). Two, three, and four plants were grown per pot for *A. mangium*, *A. mearnsii*, and *A. auriculiformis*, respectively. A microtube irrigation system supplied nutrient solution daily. The nutrient solution was the same as used to fill the pouches except for the first 6 weeks with *A. mangium* when double strength solution was used. In addition, pots with *A. mangium* and *A. mearnsii* received 3 mM starter N in the nutrient solution in the form of $CaNO_3$ for the first 3 weeks after planting. Pots were covered with sterile gravel following inoculation to help prevent rhizobial contamination.

Each of the three pot experiments was set up in a randomized complete block design with three blocks for *A. mangium* and four for *A. auriculiformis* and *A. mearnsii*.

Seedling preparation and inoculation

Seeds for all experiments were scarified and surface sterilized as described in Chapter 2. *Enterolobium cyclocarpum*, and *L. latisiliqua* were scarified in the same manner as *Albizia lebbeck*. Seeds for the pouch experiments were germinated on water agar plates except for *Albizia saman*, *A. lebbeck*, and *E. cyclocarpum*, which were germinated in expanded vermiculite. All seedlings for the pot experiments were germinated in horticultural vermiculite (Grace and Co.) and transplanted into the pots 10 to 15 days later. For *A. mangium* and *A. mearnsii*, an extra seedling was transplanted into each pot; the least vigorous seedling was removed after 5 weeks.

Rhizobial strains were grown in yeast-extract mannitol (YEM) broth (Vincent, 1970) for 8 and 11 days respectively for Pouch Experiments A and B and for 8 days for the pot experiments. Plants were inoculated 8 to 16 days after planting in pouches, and within 24 hours after planting in pots. Pouches were inoculated by applying 1 ml of undiluted inoculant supplying approximately 10^9 rhizobia ml^{-1} in Pouch Experiment A and 1 ml of inoculant diluted 10-fold supplying approximately 10^8 rhizobia ml^{-1} in Pouch Experiment B to the roots of each plant. In

pots, 1 ml of rhizobial broth culture containing approximately 10^9 rhizobia ml^{-1} was applied to each plant within 24 hours after planting and washed in with approximately 25 ml of sterile water.

Harvest and analysis

Plants were grown for various times before harvest to achieve maximal treatment differences (Table 3.2). At harvest plants in Pouch Experiment A were scored for effectiveness using a scale of 0 if no nodules formed on at least three of four replicates, "I" if plants nodulated but were not greener than uninoculated plants, "e" if at least two replicates were slightly greener than uninoculated plants, and "E" if at least two plants were greener and larger than "e" plants.

In Pouch Experiment B specificity for nodulation and effectiveness was assessed by single linkage cluster analysis of nodule numbers and shoot dry weight after standardization of values from zero to one. For shoot dry weight, uninoculated controls and treatments with values less than those of the uninoculated controls were given a value of zero. Specificity for nodulation and effectiveness was also assessed by rank correlation of nodule numbers and shoot dry weight respectively.

In the pot experiments, strains were evaluated using analysis of variance of $\log_{10}$ transformed data except for shoot dry weight of *A. auriculiformis* where the data were

not transformed because Bartlett's test showed that the variances were not significantly different at $P = 0.05$. Performance of strains used for the same species in Pouch Experiment B and in the corresponding pot experiment were compared by Spearman rank correlation.

Correlation and cluster analyses were carried out using the SYSTAT program (Wilkinson, 1990); the SAS program (SAS Institute, 1986) was used to perform analysis of variance with Tukey's HSD test in pouch experiment B the pot experiments.

RESULTS

Pouch Experiment A

In Pouch Experiment A, tree species that nodulated effectively with *Rhizobium* formed three distinct groups based on specificity for effectiveness while species that nodulated effectively with *Bradyrhizobium* formed a single group but still exibited a range of specificity for nodulation and effectiveness (Table 3.4). *Leucaena leucocephala*, *Gliricidia sepium*, and *Calliandra calothyrsus* consistently formed effective symbioses with rhizobia isolated from members of all three genera with some effective nodulation with strains from *Sesbania* species. *Sesbania grandiflora* and *Robinia pseudoacacia* only nodulated effectively with rhizobia isolated from their respective genera except for effective and

moderately effective nodulation of *S. grandiflora* with a *Calliandra* strain (TAL 1801) and an *Acacia mangium* strain (TAL 1867) respectively and moderately effective nodulation of *R. pseudoacacia* with TAL 1145 and a strain from *Calliandra* (TAL 1455). *R. pseudoacacia* nodulated ineffectively with 19 of 35 strains, including both *Rhizobium* and *Bradyrhizobium*.

A. auriculiformis, *A. mangium*, *A. mearnsii*, *Paraserianthes falcataria*, and *Tephrosia candida* nodulated effectively only with *Bradyrhizobium* strains but revealed a range of specificity for nodulation and effectiveness. *P. falcataria* and *T. candida* formed effective symbioses with all *Bradyrhizobium* strains applied except TAL 102. At the other extreme, *A. mangium* failed to nodulate with five of 12 *Bradyrhizobium* strains and nodulated ineffectively with four of the remaining strains. All species that nodulated effectively with *Bradyrhizobium* strains nodulated ineffectively with some of the *Rhizobium* strains.

Pouch Experiment B

Distinct differences in the performance of different species were observed in Pouch Experiment B with respect to nodule numbers and shoot dry weight. *Albizia saman*, *Enterolobium cyclocarpum*, and *Vigna unguiculata* were not included in species comparisons due to lack of significant differences ($P < 0.05$) between the shoot dry weight of the

uninoculated control and that of the best strain as evaluated by Tukey's HSD test. *M. atropurpureum* (Figure 3.1), *A. auriculiformis* (Figure 3.1), and *A. lebbeck* (Figure 3.3), had a higher proportion of relatively effective strains compared with *A. mangium* and *A. mearnsii* (Figure 3.2) as evaluated by shoot dry weight. *L. latisiliqua* (Figure 3.3) and *P. falcataria* (Figure 3.4) were intermediate between these groups. *A. mangium* (Figure 3.2) and *L. latisiliqua* (Figure 3.3) did not form nodules with three and four strains respectively. All other species nodulated with all 34 strains. The three strains that produced the highest shoot dry weight on *A. mearnsii* were all homologous, as was the best strain on *A. mangium*.

Single-linkage cluster analyses were used to summarize these relationships (Figure 3.2). With respect to nodulation, there were no differences discernable between *M. atropurpureum*, *A. auriculiformis*, and *A. mearnsii*. The other species were separated from this group by regularly increasing intervals, with *A. mangium* being furthest apart. Similarly, whereas *A. mearnsii* had significant rank correlation with *M. atropurpureum* and *A. auriculiformis* with respect to nodule numbers, nodulation of *A. mangium* was not significantly correlated with nodulation of any other species (Table 3.5).

DISCUSSION

Effective cross nodulation of *G. sepium*, *C. calothyrsus*, and *L. leucocephala*, by rhizobia from each of the three species but failure to nodulate effectively with most rhizobia isolated from other legumes, indicates that *G. sepium*, *C. calothyrsus*, and *L. leucocephala* belong to a common effectiveness group. Previously, *L. leucocephala* had been reported to nodulate with a strain isolated from *C. calothyrsus* (Halliday and Somasegaran, 1983) but without any information on effectiveness. Other researchers have reported effective nodulation of *G. sepium* (Somasegaran et al., 1989) and *G. maculata* (Akkasaeng et al., 1986) with fast-growing rhizobial strains. Our data conflict with unsubstantiated reports that *G. sepium* (Date, 1977; Dreyfus et al., 1987; Peoples et al., 1989; Trinick, 1982) and *Calliandra* (Peoples et al., 1989) nodulate with "cowpea," "slow-growing" or *Bradyrhizobium* strains.

The results from Pouch Experiment A support reports that *S. grandiflora* has highly specific rhizobial requirements (Abdel Magid et al., 1988; Johnson and Allen, 1952; Ndoye et al., 1990; Trinick, 1982). The data support reports of specificity for effectiveness of *R. pseudoacacia* (Allen and Allen, 1981; NifTAL and FAO, 1984). They indicate that *R. pseudoacacia* nodulates with a broad range of rhizobia in support of some reports

(Burton, 1977; Crow et al., 1981; Wilson, 1944) but in disagreement with the conclusions of Allen and Allen (1981).

Based on the pattern of effective nodulation in Pouch Experiment A and on cluster analyses and rank correlation of shoot dry weight and nodule numbers in Pouch Experiment B, *A. auriculiformis*, *A. lebbeck*, *P. falcataria*, and *T. candida* appear to be promiscuous for both nodulation and effectiveness while *A. mearnsii* is promiscuous for nodulation but specific for effectiveness, and *A. mangium* and *L. lysiliqua* are specific for both nodulation and effectiveness. These data support the findings of Allen and Allen (1939) for *A. lebbeck* and of Galiana et al. (1990) for *A. mangium*, but are in disagreement with an unsubstantiated report that *A. lebbeck* is specific in its rhizobial requirements (Duhoux and Dommergues, 1985).

The results suggest that species-specific inoculants should be developed for *S. grandiflora*, *R. pseudoacacia*, *A. mangium*, *A. mearnsii*, and *L. latisiliqua*. A common inoculant could be developed for *L. leucocephala*, *G. sepium*, and *C. calothyrsus*, an expansion of the host range suggested by Somasegaran et al. (1989).

Similarly, a single inoculant could be developed for use with all species that are promiscuous for both nodulation and effectiveness. However, because no strains emerged as distinctly superior for all of the species in

this category used in pouch experiment B and because a strain commonly recommended for inoculating grain and forage legumes in the cowpea miscellany, TAL 309 (CB 756), failed to nodulate *A. auriculiformis* effectively in pots, it would seem prudent to develop individual inoculants for each species. Another approach would be to develop a group inoculant consisting of a mixture of highly effective strains for each species rather than to rely on inoculant developed for use with other legumes that are promiscuous for both nodulation and effectiveness such as *M. atropurpureum* or *Vigna unguiculata*.

The data suggest that MPN assays conducted with *L. leucocephala*, *G. sepium* or *C. calothyrsus* should provide a reasonable approximation of the population of rhizobia present capable of nodulating the other two species. Similarly, *M. atropurpureum* will likely provide a reasonable estimate of the rhizobial densities of *A. auriculiformis*, *A. mearnsii*, *A. lebbeck*, *P. falcataria*, and *T. candida*, but not for *A. mangium* and *L. latisiliqua* due to greater specificity of the latter species for nodulation.

In keeping with view that species that are more specific for effectiveness are more likely to respond to inoculation in the field than species with less specific rhizobial requirements (Dommergues, 1987), *L. leucocephala*, *G. sepium*, *C. calothyrsus*, *A. mearnsii*, *A.*

mangium, and *L. latisiliqua* should be more likely to respond to inoculation than *A. auriculiformis*, *A. lebbeck*, *P. falcataria*, or *T. candida*.

In summary, the trees studied in the experiments described here can be categorized based on effective nodulation with *Rhizobium* or *Bradyrhizobium* and specificity for nodulation and effectiveness (Table 3.7).

Table 3.1

Conflicting reports of rhizobial affinities with tree legumes.

Genus or Species	Effective Nodulation with:	
	Bradyrhizobium	***Rhizobium***
Albizia lebbeck	Allen and Allen, 1939*	Duhoux and Dommergues, 1985
Calliandra	Peoples et al., 1989	P. Somasegaran, pers. comm.
Desmanthus	Date, 1977 and 1982 Date and Halliday, 1982 Trinick, 1982	Date, 1991* Davis, 1982*
Gliricidia	Date, 1977 Dreyfus et al., 1987 Peoples et al., 1989 Trinick, 1982	Akkasaeng et al., 1986* Somasegaran et al., 1989*
Samanea (*Albizia*)	Allen and Allen, 1939*	Gibson et al., 1982

* supporting data presented in report

Table 3.2. Plant combinations and time from inoculation to harvest for each combination.

Experiment	Plant combinations	Days to harvest
Pouch A	*Acacia auriculiformis - A. mangium*	62
Pouch A	*Acacia mearnsii*	49
Pouch A	*Calliandra calothyrsus - Paraserianthes falcataria*	47
Pouch A	*Gliricidia sepium - Lysiloma latisiliqua*	39
Pouch A	*Robinia pseudoacacia*	49
Pouch A	*Sesbania sesban - Tephrosia candida*	32
Pouch B	*Acacia auriculiformis - Albizia lebbeck*	88
Pouch B	*Acacia mangium - A. mearnsii*	74
Pouch B	*Albizia saman - Enterolobium cyclocarpum*	92
Pouch B	*L. latisiliqua - P. falcataria*	87
Pouch B	*Macroptilium atropurpureum - Vigna unguiculata*	38
Pot A	*A. auriculiformis*	101
Pot B	*A. mangium*	73
Pot C	*A. mearnsii*	59

Table 3.3

Strains used in pouch and pot experiments.

Strain (TAL No.)	Original Host	Rhizobial Genus[a]	Other Name[b]	Exper-iments[c]
7	*Gliricidia sepium*	*Rhizobium*	-	A
22	*Phaseolus lunatus*	*Bradyrhizobium*	-	A,B,C
33	*Calliandra calothyrsus*	*Rhizobium*	-	A
45	*Paraserianthes falcataria*	*Bradyrhizobium*	-	A,B,C
47	*Enterolobium cyclocarpum*	*Bradyrhizobium*	-	B,C
60	*Enterolobium cyclocarpum*	*Bradyrhizobium*	-	B
63	*Acacia mearnsii*	*Bradyrhizobium*	Nit 1B2	B,C
69	*Erythrina indica*	*Bradyrhizobium*	Nit 47A1	B,C
82	*Leucaena leucocephala*	*Rhizobium*	-	A
102	*Glycine max*	*Bradyrhizobium*	USDA 110	B
111	*Acacia mearnsii*	*Bradyrhizobium*	-	B,C
112	*Acacia mearnsii*	*Bradyrhizobium*	-	C
126	*Acacia mearnsii*	*Bradyrhizobium*	-	C
132	*Acacia mearnsii*	*Bradyrhizobium*	-	C
133	*Acacia mearnsii*	*Bradyrhizobium*	-	C
134	*Acacia mearnsii*	*Bradyrhizobium*	-	C
169	*Vigna unguiculata*	*Bradyrhizobium*	Nit 176A22	C
173	*Vigna unguiculata*	*Bradyrhizobium*	Nit 176A30	B,C
179	*Acacia mearnsii*	*Bradyrhizobium*	-	C
180	*Acacia mearnsii*	*Bradyrhizobium*	-	C
201	*Canavalia ensiformis*	*Bradyrhizobium*	Nit 22A4	B
209	*Vigna radiata*	*Bradyrhizobium*	-	B
309	*Macrotyloma africanum*	*Bradyrhizobium*	CB 756	C
363	*Albizia lebbeck*	*Bradyrhizobium*	-	A
569	*Desmodium uncinatum*	*Bradyrhizobium*	MAR 472	B,C
582	*Leucaena leucocephala*	*Rhizobium*	CB 81	A
583	*Leucaena leucocephala*	*Rhizobium*	NGR 8	A
644	*Phaseolus acutifolius*	*Bradyrhizobium*	CIAT 257	B,C
651	*Calopogonium mucunoides*	*Bradyrhizobium*	UMKL 44	B,C
658	*Stylosanthes sp.*	*Bradyrhizobium*	CIAT 71	B
749	*Erythrina indica*	*Bradyrhizobium*	CIAT 35	B,C
795	*Tephrosia glauca*	*Bradyrhizobium*	CIAT 496	A
833	*Samanea saman*	*Bradyrhizobium*	UMKL 27	B,C
850	*Crotalaria sp.*	*Bradyrhizobium*	UMKL 71	B,C
881	*Acacia koa*	*Bradyrhizobium*	-	B,C
940	*Acacia mearnsii*	*Bradyrhizobium*	Num 777	C
941	*Acacia mearnsii*	*Bradyrhizobium*	Num 778	C
942	*Acacia mearnsii*	*Bradyrhizobium*	Num 779	C
1000	*Arachis hypogaea*	*Bradyrhizobium*	-	B
1119	*Sesbania sp.*	*Rhizobium*	IC 91	A
1122	*Albizia stipulata*	*Bradyrhizobium*	NGR 143	B
1137	*Sesbania sp.*	*Rhizobium*	CIAT 175	A
1145	*Leucaena leucocephala*	*Rhizobium*	CIAT 1967	A
1280	*Samanea saman*	*Bradyrhizobium*	ALLEN 708	B

Table 3.3 (Continued)

Strains used in pouch and pot experiments.

1371	*Arachis hypogaea*	*Bradyrhizobium*	Nit 8A11	B
1380	*Crotalaria paulina*	*Bradyrhizobium*	Nit 32H1	A,B,C
1384	*Acacia mearnsii*	*Bradyrhizobium*	-	C
1385	*Acacia mearnsii*	*Bradyrhizobium*	-	C
1386	*Acacia mearnsii*	*Bradyrhizobium*	-	C
1387	*Acacia mearnsii*	*Bradyrhizobium*	-	C
1388	*Acacia mearnsii*	*Bradyrhizobium*	-	B,C
1389	*Acacia mearnsii*	*Bradyrhizobium*	-	C
1446	*Acacia auriculiformis*	*Bradyrhizobium*	-	A,B,C
1449	*Acacia auriculiformis*	*Bradyrhizobium*	-	C
1450	*Acacia auriculiformis*	*Bradyrhizobium*	-	C
1455	*Calliandra surinamensis*	*Rhizobium*	-	A
1457	*Acacia albida*	*Bradyrhizobium*	-	B
1521	*Acacia auriculiformis*	*Bradyrhizobium*	-	A,B
1522	*Acacia auriculiformis*	*Bradyrhizobium*	-	C
1530	*Enterolobium cyclocarpum*	*Bradyrhizobium*	-	B,C
1536	*Albizia lebbeck*	*Bradyrhizobium*	-	A,B,C
1597	*Albizia lebbeck*	*Bradyrhizobium*	-	B,C
1770	*Gliricidia sepium*	*Rhizobium*	-	A
1779	*Sesbania grandiflora*	*Rhizobium*	-	A
1788	*Gliricidia maculata*	*Rhizobium*	-	A
1801	*Calliandra calothyrsus*	*Rhizobium*	BR 4301	A
1806	*Gliricidia sepium*	*Rhizobium*	BR 8801	A
1829	*Sesbania rostrata*	*Azorhizobium*	ORS 571	A
1852	*Albizia caribaea*	*Bradyrhizobium*	-	B
1867	*Acacia mangium*	*Bradyrhizobium*	LB 5	A,B,C
1869	*Acacia mangium*	*Rhizobium*	LB 7	A
1883	*Flemingia macrophylla*	*Bradyrhizobium*	Nit 52A1	A,B
1886	*Sesbania longifolia*	*Rhizobium*	Nit 145B1	A
1887	*Leucaena leucocephala*	*Rhizobium*	MS 111	A
1908	*Glycine max*	*Bradyrhizobium*	USDA 94	B

[a] determined by IPTG XGal assay (Sambrook et al., 1989) in conjunction with growth on sucrose and lactose (Appendix A).

[b] strains with no other name were isolated by the NiFTAL Project.

[c] A = Pouch Experiment A, B = Pouch Experiment B, C = pot experiments.

For pot experiments, homologous strains were used only on the original host except for TAl nos. 1446, 1388, 63, and 111 which were used in all three pot experiments. TAL 309 and TAL 850 were not used in the *A. mangium* pot experiment.

Table 3.4. Effectiveness of *Rhizobium* and *Bradyrhizobium* strains on seven tree legumes.

Rhizobial Strains		Tree Species									
TAL No.	Original Host	Ll	Gs	Cc	Sg	Rp	Aa	Am	Ame	Pf	Tc
1887	*Leucaena leucocephala*	E	E	e	0	I	0	I	I	I	0
583	*Leucaena leucocephala*	E	E	e	0	I	0	I	I	0	np
1145	*Leucaena leucocephala*	E	E	e	0	e	0	0	0	I	I
582	*Leucaena leucocephala*	e	I	I	0	I	I	I	I	0	0
82	*Leucaena leucocephala*	e	E	e	0	0	0	I	0	0	0
7	*Gliricidia sepium*	E	E	E	0	I	I	I	0	I	0
1788	*Gliricidia maculata*	E	E	E	I	I	I	0	0	I	I
1806	*Gliricidia sepium*	E	E	E	0	I	0	0	0	I	I
1770	*Gliricidia sepium*	E	E	E	0	I	0	0	0	I	0
1801	*Calliandra calothyrsus*	e	E	0	E	I	I	I	0	0	0
33	*Calliandra calothyrsus*	E	E	E	0	I	I	0	0	I	I
1455	*Calliandra surinamensis*	E	E	E	0	e	0	0	0	I	I
1779	*Sesbania grandiflora*	0	0	0	E	I	0	I	0	I	0
1886	*Sesbania longifolia*	e	E	I	E	I	0	I	0	I	0
1137	*Sesbania* sp.	e	E	E	0	0	0	I	I	I	I
1114	*Sesbania* sp.	0	0	0	E	I	0	0	0	0	np
1119	*Sesbania* sp.	0	0	0	E	0	0	0	0	0	0
1829	*Sesbania rostrata*	0	0	e	I	I	I	I	0	I	I
183	*Robinia pseudoacacia*	0	0	0	0	E	0	0	0	0	np
1889	*Robinia pseudoacacia*	0	0	0	0	E	0	0	0	0	np
1907	*Robinia pseudoacacia*	0	0	0	0	E	0	0	0	0	np
1869	*Acacia mangium*	e	I	e	I	I	I	I	e	0	0
1867	*Acacia mangium*	0	0	0	e	0	E	E	e	E	E
1446	*Acacia auriculiformis*	0	0	0	0	0	E	0	E	E	E
1521	*Acacia* sp.	0	0	0	0	I	E	0	e	E	E
45	*Paraserianthes falcataria*	0	I	0	I	I	?	E	E	E	E
363	*Albizia lebbeck*	0	0	0	0	0	0	I	I	e	E
1536	*Albizia lebbeck*	0	I	0	0	I	E	np	I	E	np
795	*Tephrosia glauca*	0	0	0	0	0	0	0	I	E	E
1883	*Flemingia macrophylla*	0	0	0	0	0	E	I	0	e	E
22	*Phaseolus lunatus*	0	0	0	0	0	E	E	e	E	np
1380	*Crotalaria paulina*	0	I	0	0	0	E	I	E	E	E
1000	*Arachis hypogaea*	0	0	0	0	0	E	0	e	e	np
1908	*Glycine max*	0	I	0	0	I	e	I	I	e	E
102	*Glycine max*	0	0	0	0	I	0	0	I	I	np

Effectiveness code: 0 = no nodules, e = moderately effective, I = ineffective, E = effective, ? = inconsistent response, np = not performed.
Legume species: Ll = *Leucaena leucocephala*, Gs = *Gliricidia sepium*, Cc = *Calliandra calothyrsus*, Sg = *Sesbania grandiflora*, Rp = *Robinia pseudoacacia*, Aa = *Acacia auriculiformis*, Am = *Acacia mangium*, Ame = *Acacia mearnsii*, Pf = *Paraserianthes falcataria*, Tc = *Tephrosia candida*

= *Leucaena-Gliricidia-Calliandra Rhizobium* group
= *Sesbania Rhizobium* group
= *Robinia Rhizobium* group
= *Bradyrhizobium* group

Table 3.5. Comparison of species used in Pouch Experiment B on the basis of rank correlation of shoot dry weight (A) and nodule numbers (B) produced by individual rhizobial strains.

A. Shoot Dry Weight

	Ma[a]	Aa	Am	Ame	Al	Ll
	-Spearman rank correlation coefficients-					
Aa	0.402*[b]					
Am	0.374*	0.328*				
Ame	0.189	0.192	0.530**			
Al	0.375*	0.316*	0.230	0.366*		
Ll	0.366*	0.372*	0.314*	0.090	0.164	
Pf	0.528**	0.238	0.019	0.086	0.693**	0.296

B. Nodule Numbers

	Ma	Aa	Am	Ame	Al	Ll
	-Spearman Rank Correlation Coefficients-					
Aa	0.212					
Am	0.209	0.269				
Ame	0.304*	0.445**	0.149			
Al	0.094	0.277	0.148	0.320*		
Ll	0.300*	0.289	0.109	0.059	-0.294	
Pf	0.020	-0.159	-0.258	-0.404	0.104	0.094

[a] Legume species: Ma = *M. atropurpureum*, Aa = *A. auriculiformis*, Am = *A. mangium*, Ame = *A. mearnsii*, Al = *Albizia lebbeck*, Ll = *Lysiloma latisiliqua*, Pf = *P. falcataria*.
[b] *, ** = significant at $P < 0.05$ and $P < 0.01$, by 1 tailed test

Table 3.6. Comparison of species used in Pouch Experiment B with the same species used in the pot experiments by rank correlation of shoot dry weight and nodule numbers for strains used in both experiments.

Spearman rank correlation coefficients				
Shoot dry weight			Nodule number	
A. auriculiformis	*A. mangium*	*A. mearnsii*	*A. mangium*	*A. mearnsii*
0.25	0.72***	0.67***	0.72***	0.80***

*** = significant at $P < 0.001$

Table 3.7

Rhizobial Specificity of Tree Legumes.

	Specificity*	
Trees nodulating effectively with *Rhizobium*	**Nodulation**	**Effectiveness**
Leucaena leucocephala, Gliricidia sepium, Calliandra calothyrsus	S	S
Sesbania grandiflora	S	S
Robinia pseudoacacia	P	S
Trees nodulating effectively with *Bradyrhizobium*		
Acacia auriculiformis, Albizia lebbeck, Paraserianthes falcataria, Tephrosia candida	P	P
Acacia mearnsii	P	S
Acacia mangium	S	S
Lysiloma latisiliqua	S	S

*S = Specific, P = Promiscuous

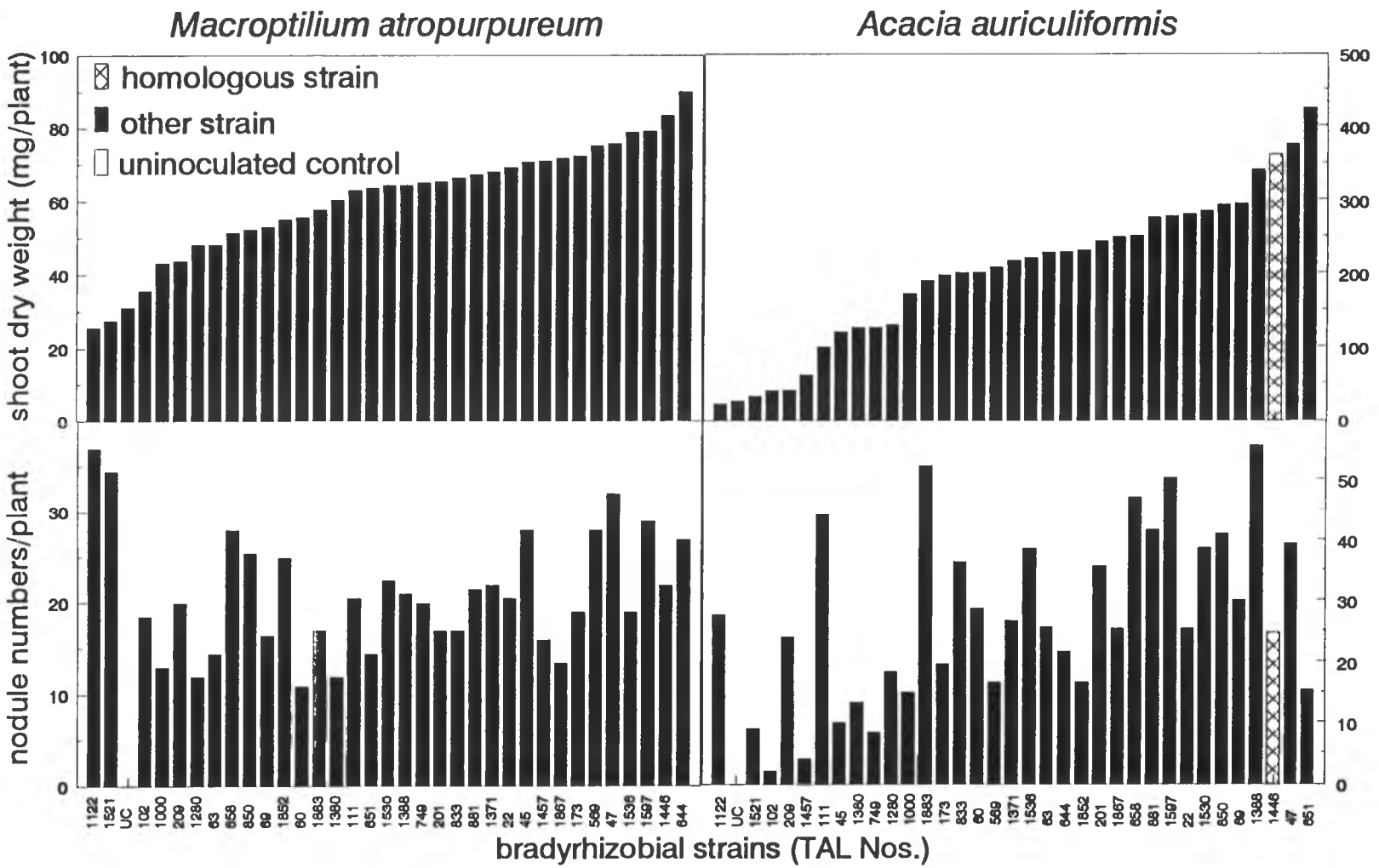


Figure 3.1. Nodule numbers and shoot dry weight of M. atropurpureum and Acacia auriculiformis inoculated with 34 bradyrhizobial strains in Pouch Experiment B.

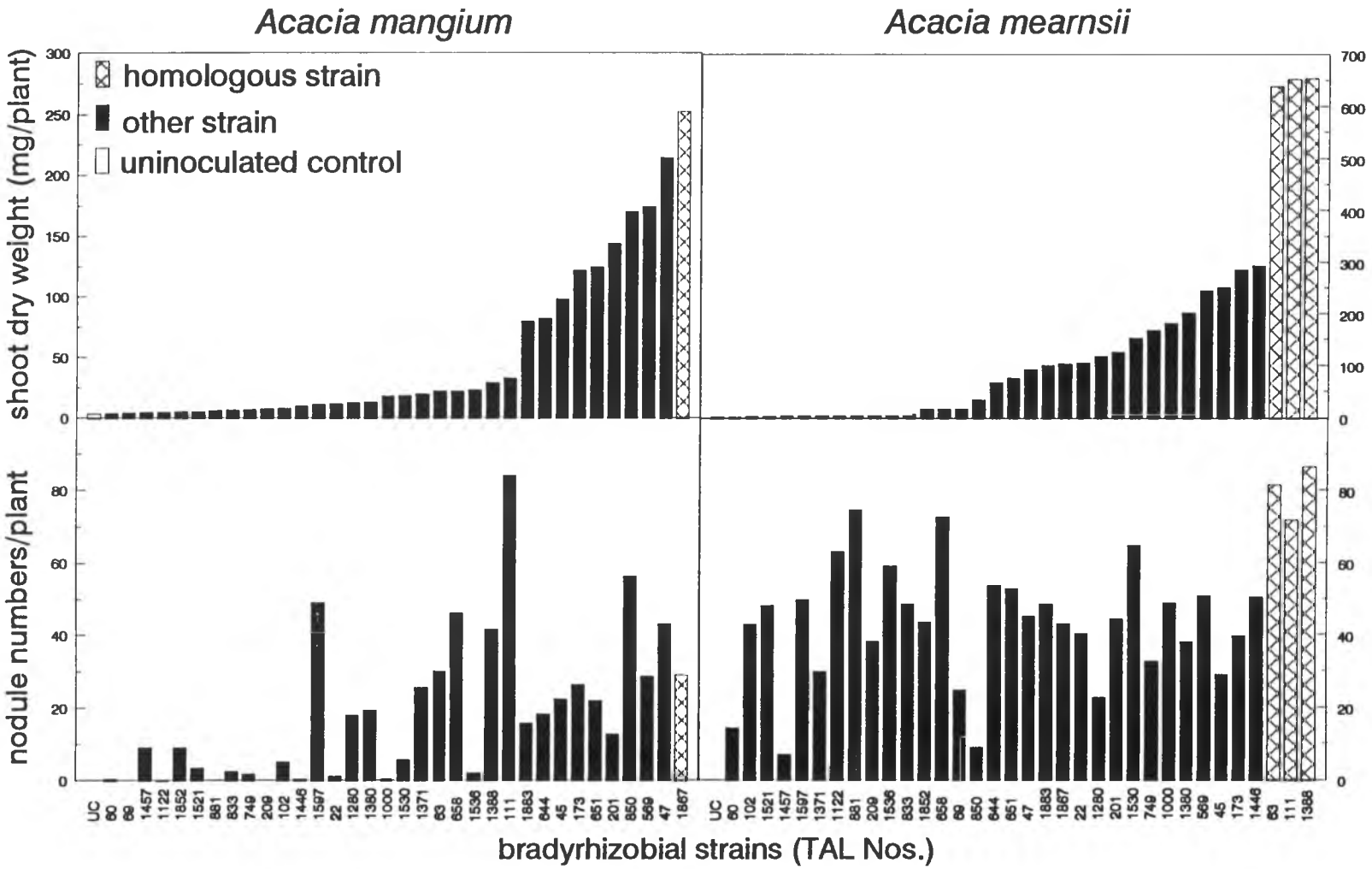


Figure 3.2. Nodule numbers and shoot dry weight of A. mangium and A. mearnsii inoculated with 34 bradyrhizobial strains in Pouch Experiment B.

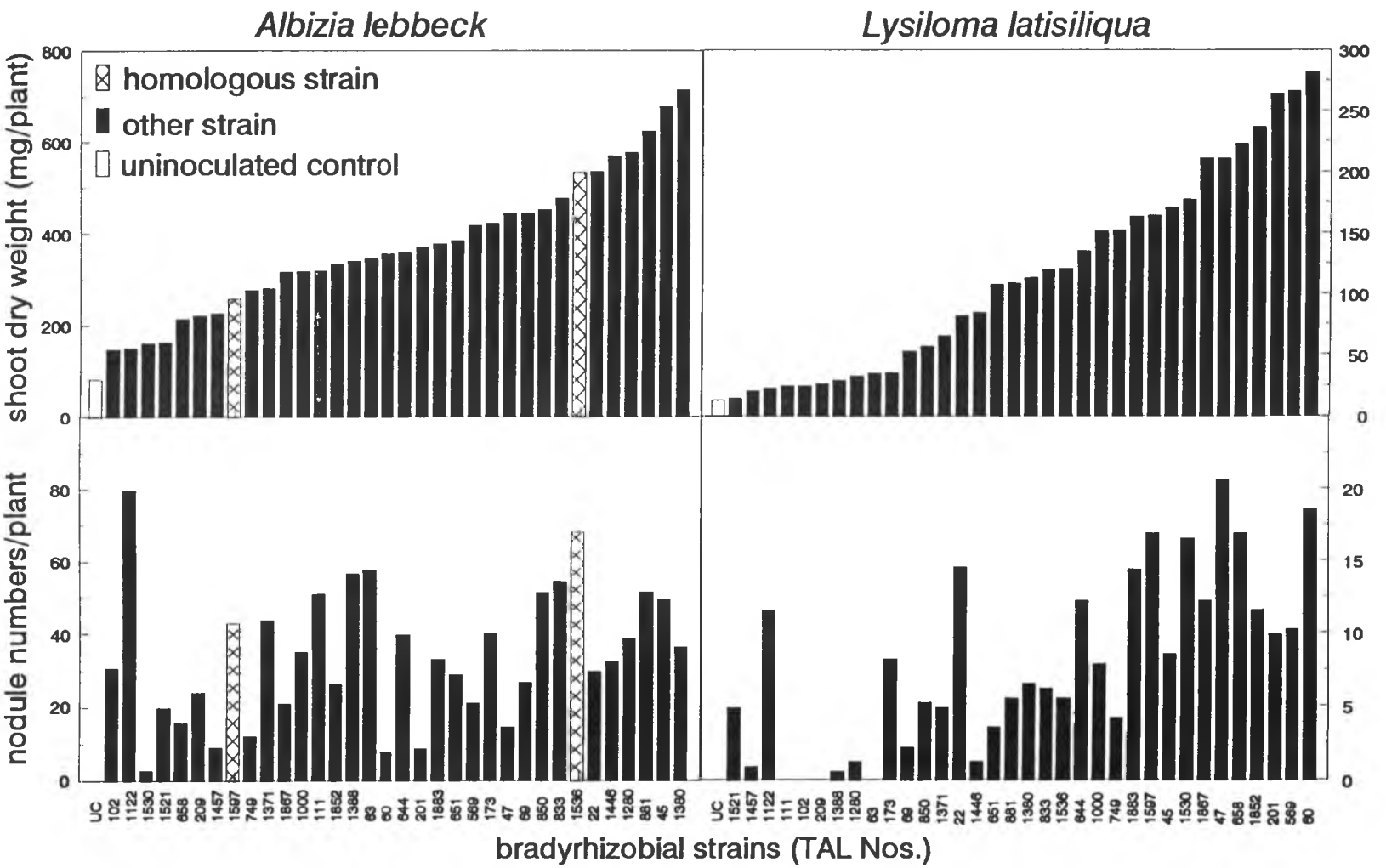


Figure 3.3. Nodule numbers and shoot dry weight of A. lebbeck and L. latisiliqua inoculated with 34 bradyrhizobial strains in Pouch Experiment B.

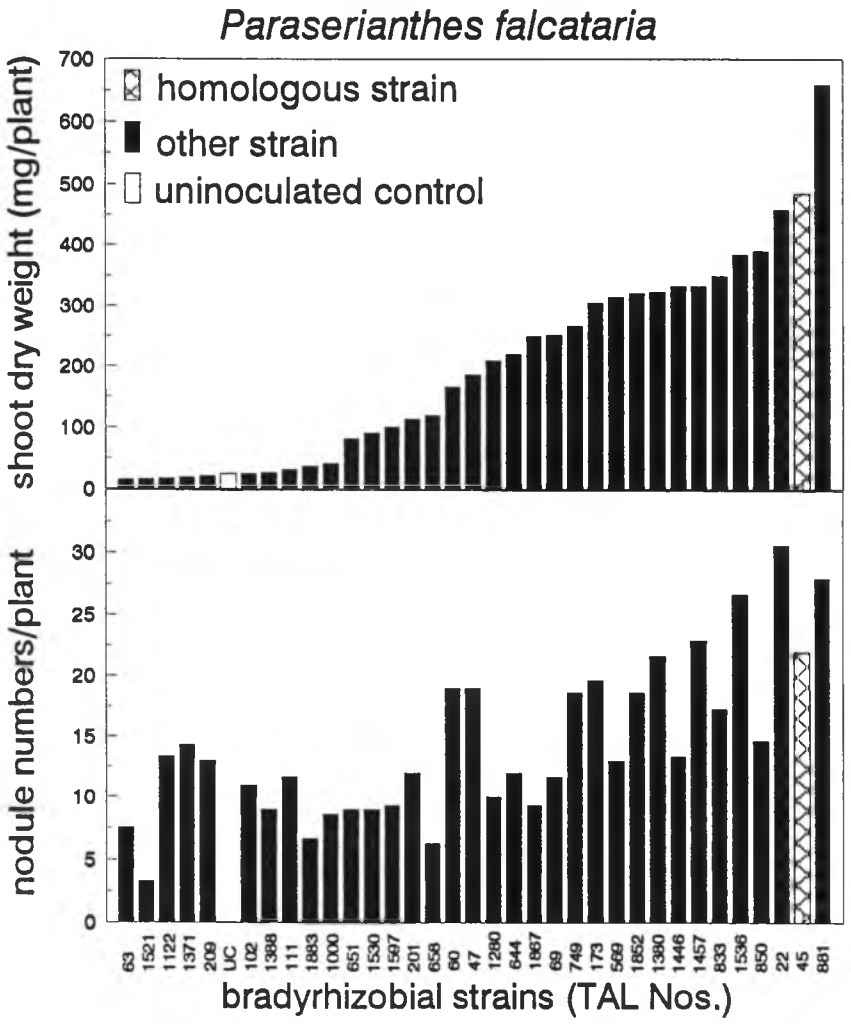


Figure 3.4. Nodule numbers and shoot dry weight of Paraserianthes falcataria inoculated with 34 bradyrhizobial strains in Pouch Experiment B.

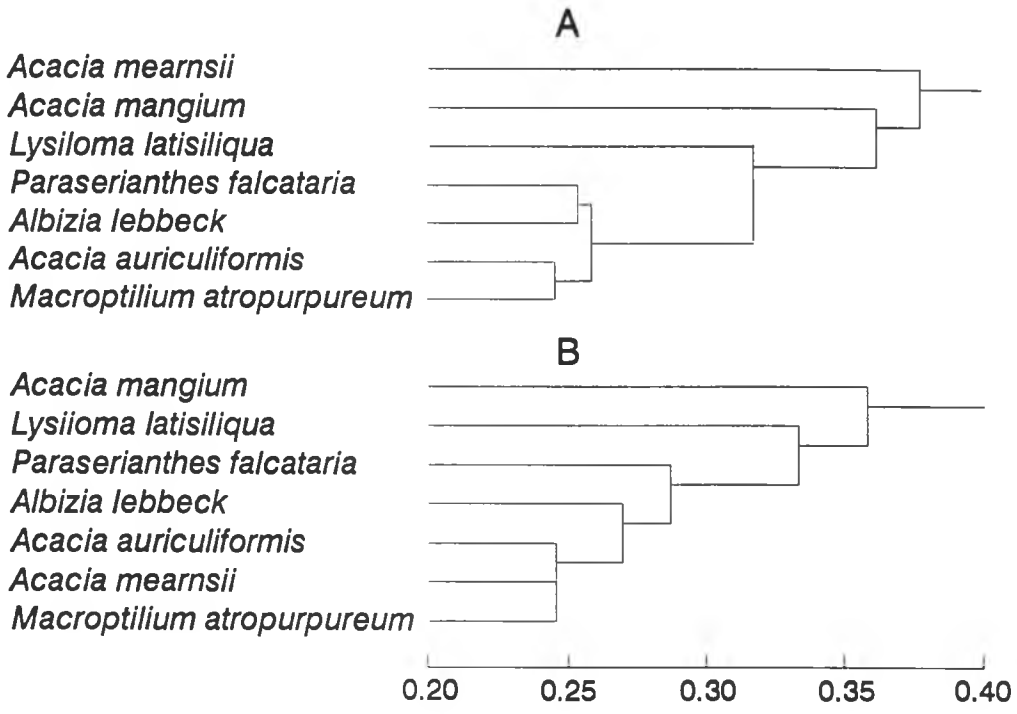


Figure 3.5. Single linkage cluster analysis by Euclidean distance of shoot dry weight (A) and nodule numbers (B) of legume species from Pouch Experiment B.

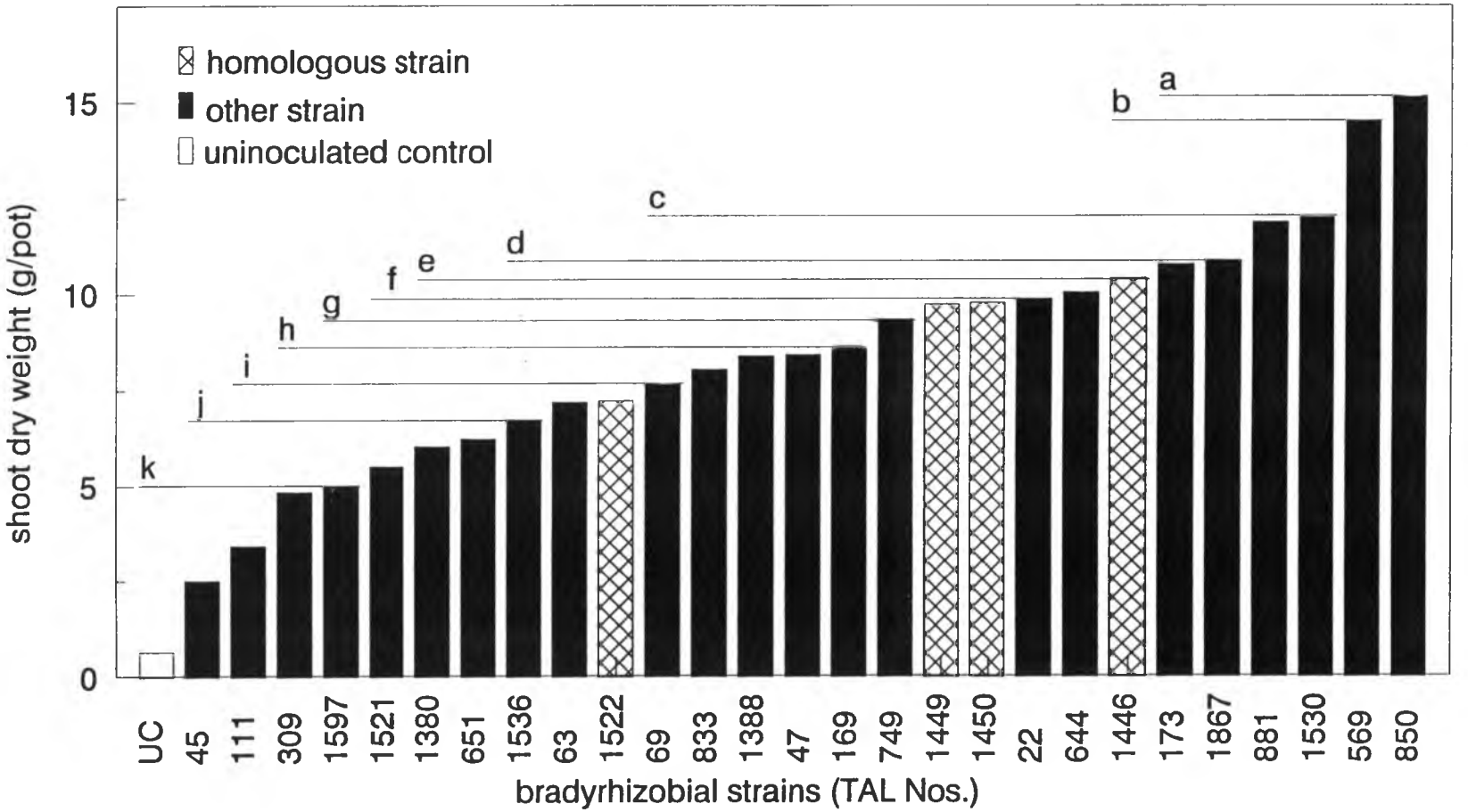


Figure 3.6. Shoot dry weight of Acacia auriculiformis inoculated with 27 bradyrhizobial strains in a pot experiment. Bars under a line are not significantly different ($P < 0.05$) by Tukey's HSD test.

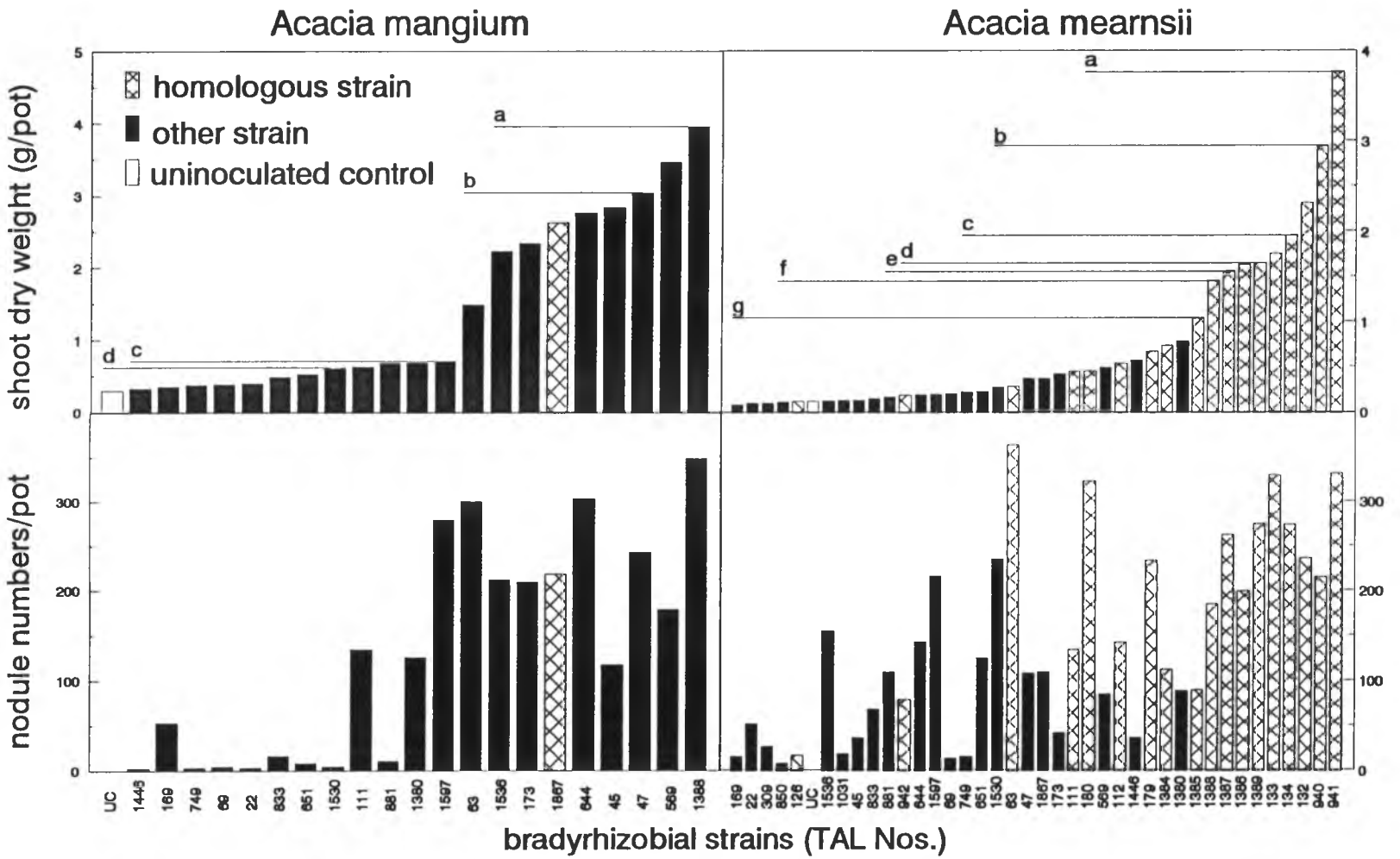


Figure 3.7. Shoot dry weight and nodule numbers of A. mangium and A. mearnsii from pot experiments. Bars under a line are not significantly different ($P < 0.05$) by Tukey's HSD test.

Chapter 4

Response of Tree Legumes to Rhizobial Inoculation in Relation to the Population Density of Indigenous Rhizobia

ABSTRACT

To determine the relationship between yield response to inoculation and rhizobial population density for leguminous trees, inoculation experiments were conducted in pots containing four soils in a greenhouse on the island of Maui, Hawaii, with *Acacia auriculiformis*, *A. mangium*, *A. mearnsii*, *Leucaena diversifolia*, *Robinia pseudoacacia*, and *Sesbania grandiflora*. Densities of indigenous rhizobia were determined by most-probable-number plant-infection assays. Response to inoculation was calculated for each species-soil combination as the increase in shoot nitrogen due to inoculation. The magnitude of the response was inversely related to the density of rhizobia in the soil, with the greatest responses where rhizobial densities were less than 50 rhizobia g^{-1} soil. Tree species known to nodulate effectively with *Rhizobium* had a higher proportion of significant responses to inoculation than species known to nodulate effectively with *Bradyrhizobium*. *R. pseudoacacia* and *A. mearnsii* responded significantly ($P < 0.05$) to inoculation in three and one soils respectively despite the presence of more than 1000 rhizobia g^{-1} soil. A

hyperbolic model best described the relationship of the increase in shoot dry weight due to rhizobial inoculation to the density of indigenous rhizobia. Incorporation of an index of available soil N into this model resulted in models with lower residual mean square values, indicating the important role of mineral N in mediating responses to rhizobial inoculation.

INTRODUCTION

As natural forests are depleted and fallow periods diminished in shifting agricultural systems of the tropics, fast-growing nitrogen-fixing trees are becoming more important as sources of fuelwood, fodder, and nitrogen-rich biomass. Inoculation of tree seeds or seedlings with rhizobia can insure that enough highly effective rhizobia are present to satisfy the trees' demand for biological nitrogen fixation (BNF). Where available mineral N limits growth and appropriate indigenous rhizobia are scarce or absent, inoculation can increase yields and and monetary returns. However, infrastructure and educational constraints often prevent farmer use of rhizobial inoculant in the tropics. Because of the expense involved in overcoming these constraints, knowledge of if, and how much, inoculation is likely to increase yields can help farmers and regional planners make sound decisions concerning investment in rhizobial

inoculant technology. In this paper, a method is evaluated for predicting the magnitude of response to inoculation for six tree legumes based on the density of indigenous rhizobia.

The quantification of factors regulating the response of legumes to inoculation has been proposed as an approach for assessing the magnitude of responses to inoculation with rhizobia without resorting to pot or field experiments (Singleton and Tavares, 1986; Brockwell et al., 1988). Major factors likely to influence response to inoculation are (i) limitations to plant growth other than N; (ii) density of indigenous rhizobia in soil; (iii) effectiveness of indigenous rhizobia; and (iv) available mineral N (Singleton et al., 1991a). A major advantage of this approach is that it permits extrapolation of results beyond the fields where soils were tested.

Models of the response to inoculation of field-grown grain and forage legumes (Thies, 1991b) indicated that when limitations other than N are removed, rhizobial density as estimated by the MPN assay is the primary factor determining the magnitude of response to inoculation. Models that accounted for the effects of mineral N availability in addition to rhizobial density improved the agreement between observed and predicted inoculation responses. Singleton et al. (1991a) noted that measures of indigenous rhizobial population

effectiveness have not proven to be good indicators of the magnitude of response to inoculation.

This study was conducted to determine the relationship of shoot N response to rhizobial inoculation of six tree legumes to an index of available mineral N and to the density of their indigenous rhizobial populations. A second purpose was to evaluate how specificity for nodulation and effectiveness of tree legumes (Chapter 3) relate to patterns of indigenous rhizobial densities and responses to inoculation.

MATERIALS AND METHODS

General Experimental Approach

Pot experiments were conducted in a greenhouse at 110 m elevation at Hamakuapoko, Maui, Hawaii. Four soils (Table 4.1) were selected, two with total indigenous rhizobial densities greater than 10 000 rhizobia g^{-1} soil, and two with rhizobial densities less than 200 rhizobia g^{-1} soil, as determined by MPN counts on five legumes representing the cross-inoculation groups of the legumes growing at the sites (Woomer et al., 1989a). *Leucaena leucocephala* was growing at one low rhizobial density site and at one high density site. Tree species were selected on the basis of rhizobial specificity as determined in Chapter 3. Three species known to nodulate with *Rhizobium*, *Leucaena diversifolia*, *Robinia pseudoacacia*,

and *Sesbania grandiflora*, were from genera determined in Chapter 3 to belong to distinct effectiveness groups. The three species chosen that nodulate effectively with *Bradyrhizobium*, *Acacia auriculiformis*, *A. mangium*, and *A. mearnsii*, showed a range of specificity for effectiveness and infectiveness (Chapter 3).

Seed sources were the same as indicated in Chapter 2. *Macroptilium atropurpureum* cv. siratro, a herbaceous forage legume which nodulates effectively with many *Bradyrhizobium* (Vincent, 1970) was included for comparison with the *Acacia* spp. Treatments were (i) inoculation with rhizobia and (ii) no inoculation. Two species in each soil received mineral N to measure yield potential in the growth system. The experiment was divided into two. The trials in Pane and Makawao soils were conducted from 7 December 1990 to 8 February 1991 and in Keahua and Waiakoa soils from 1 February 1991 to 5 April 1991. Pots were arranged in a randomized complete block design, with four replicates. Results were analysed using SYSTAT, version 5.0 (Wilkinson, 1990), by dependent one-tailed t tests for each species-soil combination.

Soil Collection and Plant Culture

At each soil collection site the soil was excavated to a depth of 20 cm and passed through a screen with a 5 mm opening. Five-liter black plastic pots lined with polyethylene bags were filled with 2000, 3300, 3500, and

4520 g of soil (dry weight basis) per pot for Pane, Makawao, Keahua, and Waiakoa soils respectively. These weights produced approximately equivalent volumes of each soil. The following fertilizers were added (Kg^{-1} soil): 1.07 g K_2HPO_4, 0.25 g $MgSO_4 7H_2O$, and 0.5 ml kg^{-1} of a liquid micronutrient mix (Hawaiian horticultural mix, Monterey Chemical Co.). A total of 450 mg of N Kg^{-1} soil as ammonium nitrate was added to the mineral nitrogen controls at three intervals: 50 mg kg^{-1} soil at the time of inoculation, 150 mg kg^{-1} soil 3 weeks after inoculation, and 150 mg kg^{-1} soil 6 weeks after inoculation.

Tree seeds were scarified and surface sterilized as described in Chapter 2. *M. atropurpureum* seeds were treated in the same manner as *L. diversifolia*, except exposure to concentrated sulfuric acid was limited to 8 minutes. Imbibed seeds were planted in trays containing expanded horticultural vermiculite (Grace and Co.) 4 to 10 days prior to transplanting into the pots. Four to eight seedlings were planted per pot and thinned to four plants per pot after 3 weeks. Following inoculation, the surface of each pot was covered with 850 g of sterile washed road gravel to guard against rhizobial contamination. Pots with *M. atropurpureum* were provided with sterile stakes because they have a climbing growth habit. Pots were watered to field capacity every 3-4 days as required throughout the course of the experiment.

Inoculation and Harvest

The inoculant consisted of a three strain mixture of appropriate strains for each species (Table 4.2). Each strain was grown separately for 6 to 10 days in either yeast-extract manitol (YEM) broth (Vincent, 1970) for the first two soils or in arabinose-gluconate medium (Sadowsky et al., 1987) for the second two soils. Within 2 days after planting, each pot received 5 ml of a 10^{-3} dilution of the appropriate three-strain mixture. The inoculum was applied evenly around the roots of each seedling to give approximately 5 x 10^{6} rhizobia per pot, an inoculation rate within recommended rates. The inoculant was washed into the soil with 50 ml of sterile water.

Plants were harvested 9 weeks after inoculation. Tops were severed at gravel level, dried at 70°C, weighed, ground, and analysed for N content using a Leco 600 C-H-N analyser. The root systems of three replicates were cleaned by washing over 1 mm mesh screens. Nodules were removed and counted, and both roots and nodules weighed after drying at 70°C.

Soil N Availability

N mineralization values for each soil were determined following the anaerobic incubation method of Keeney (1982). A standard curve was used to determine the amount of NH_4^+ present after distillation to avoid the need to standardize H_2SO_4. N mineralization values were multiplied

by the dry weight of soil per pot and the number of weeks the plants were grown to yield an index of N mineralized per pot over the course of the experiment. Demand for symbiotic N was assessed by calculating the percent N derived from fixation (%NDF) of *S. grandiflora* in each soil. For each soil, %NDF was calculated as:

$$\%NDF = \frac{(N_{SI} - N_{SU})}{N_{SI}} \times 100$$

where N_{SI} = average total shoot N of *S. grandiflora* in inoculated pots, and N_{SU} = average shoot N of *S. grandiflora* in uninoculated pots. The few nodules produced by *S. grandiflora* in uninoculated pots were completely white in cross-section except for a single plant in Pane soil. Shoot dry weight from this pot was not used for the calculation of %NDF.

MPN assays

MPN assays for each species-soil combination were conducted using the best growth system identified for each species in Chapter 2. For each of the two sets of greenhouse experiments, plate counts and MPN assays of pure rhizobial cultures (Table 4.2) were compared as described in Chapter 2 to provide an evaluation of the growth systems used, in accordance with the

recommendations of Thompson and Vincent, 1967; Scott and Porter, 1986; and Singleton et al., 1991b.

Soil for the MPN assays were taken just prior to planting from extra pots used in the pot experiment. Fifty g of soil (dry weight equivalent) was used for the initial dilutions. Dilution ratios were 4.0 for *L. diversifolia*, *R. pseudoacacia*, and *S. grandiflora* in Pane and Makawao soils and 5.0 for all other species-by-soil combinations. The amount of soil applied at the lowest dilution level was 1 g for Pane and Makawao soils, and 0.2 g for the other two soils. Eight uninoculated controls were interspersed among the growth units of each MPN assay.

Nodulation was assessed at 7 weeks after inoculation. The most-probable-number was determined with a computer program (Woomer et al., 1990), using as initial dilution the highest dilution in which all four replicates nodulated or, in the case of *A. mearnsii* in Makawao soil where no dilution level had four nodulated growth units, the third from last dilution level.

Models of Response to Inoculation

Models relating the response to inoculation to the density of indigenous rhizobia were fit to the data using a variety of functions with the 'nonlin' module of the SYSTAT program version 5.0 (Wilkinson, 1990). Response to

inoculation for each species-soil combination was defined as

$$R = \frac{(N_{SI} - N_{SU})}{N_{SU}} \times 100$$

where R = response to inoculation and N_{SI} and N_{SU} are as described for %NDF.

The indices of available mineral N and symbiotic N demand were incorporated into a mathematical equation to model the effect of mineral N and symbiotic N demand on the relationship of rhizobial density to response to inoculation. The model used was:

$$y = \frac{a}{1+MPN}$$

where y is shoot N increase due to inoculation expressed as a percentage of the shoot N of plants in uninoculated pots, a is the y-intercept, and x = 1 plus the density of the indigenous rhizobial population, expressed as the number of rhizobia g^{-1} soil on a dry weight basis. Linear, exponential, power, and hyperbolic functions of mineralized N and %NDF were incorporated into this model through replacement of the y-intercept.

Rhizobial Isolation and Effectiveness Tests

Rhizobia were isolated from at least 20 nodules of *R. pseudoacacia* in both Makawao and Pane soils. These rhizobia were selected for characterization because the

response of *R. pseudoacacia* in these two soils despite the presence of over 20 000 rhizobia g^{-1} soil suggested that the rhizobia were ineffective at fixing N_2. Random nodules from extra uninoculated pots were used for isolating the rhizobia. Rhizobia from each nodule were isolated, authenticated, and their pH reactions on bromothymol blue determined following methods described in Somasegaran and Hoben (1985).

Isolates from *R. pseudoacacia* from the two soils were inoculated onto agar slants containing legumes in the effectiveness groups of legume species nodulating with *Rhizobium* found growing at each soil collection site. The purpose was to determine the natural host(s) of the rhizobia that nodulated *R. pseudoacacia* from among the legumes growing at each site. The species used were: *Trifolium repens*, *Vicia* sp., and *Medicago sativa* in Pane soil and *T. repens* and *L. diversifolia* in Makawao soil.

An effectiveness test was conducted with 20 isolates from each soil. Isolates from each soil were grown for 7 days in YMB, mixed, and inoculated onto *R. pseudoacacia* in growth pouches (two plants/pouch) prepared as described in Chapter 3, using 1 ml of inoculum per pouch. Other pouches were inoculated in the same manner with a mixture of the three inoculant strains used in the pot experiments, or left uninoculated. Seven replicates were used per treatment in a completely randomized design.

Plants were harvested after 8 weeks. Shoot dry weight was measured and analysed by analysis of variance with each soil analysed separately.

RESULTS

MPN Assays

MPN results (Table 4.3) are species and soil specific. No rhizobia for *S. grandiflora* and *L. diversifolia* were detected in Pane soil where the MPN estimate for *R. pseudoacacia* was over 10^4 rhizobia g^{-1} soil. No rhizobia from *S. grandiflora* were detected in Makawao soil where the rhizobial densities of *R. pseudoacacia* and *L. diversifolia* were both greater than 10^4 rhizobia g^{-1} soil. All pure culture MPN estimates had plate count:MPN ratios less than 20.

Response to Mineral N and Inoculation with Rhizobia

Response to mineral N was significant ($P< 0.05$) for all applicable species-soil combinations (Table 4.4).

Response to inoculation as evaluated by total shoot nitrogen (Figure 4.1) was species and soil specific. Of 12 tree-soil combinations involving species that nodulate with *Rhizobium*, increased N accumulation was observed in 11 cases of which 9 were significant at $P < 0.05$, with another significant at $P < 0.10$. *L. diversifolia* did not respond significantly to inoculation in the two soils from sites where *L. leucocephala* occurred. Of 12 tree-soil

combinations involving species that nodulate with *Bradyrhizobium*, increased N accumulation was observed in seven cases of which *A. auriculiformis* in Waiakoa soil, *A. mangium* in Keahua soil, and *A. mearnsii* in Pane and Keahua soils were significant at $P < 0.05$. *M. atropurpureum* did not respond significantly to inoculation in any soil. Response to inoculation in terms of shoot dry weight (Figure 4.2), and nodule dry weight (Figure 4.3) were very similar to that of total shoot nitrogen: 13 and 11 total responses were significant respectively. Increased root weight (Figure 4.2), significant in 8 cases, and nodule numbers (Figure 4.3), significant in seven cases were not as good indicators of response to inoculation. Correlation coefficients of total shoot nitrogen with shoot mass, nodule mass, root mass, and nodule number were significant ($P < 0.05$): 0.98, 0.88, 0.51, and 0.43, respectively.

Significant shoot nitrogen responses to inoculation occurred in eight out of nine cases where the rhizobial population was less than 50 rhizobia g^{-1} soil. Where the population was greater than 50 rhizobia g^{-1} soil, significant responses were observed in six out of 14 cases. Those species that responded where the MPN estimate was greater than 50 rhizobia g^{-1} soil were *R. pseudoacacia* in Pane, Makawao, and Waiakoa soils, *A. mearnsii* in Pane and Keahua soils, and *A. auriculiformis*

in Waiakoa soil. The mean shoot nitrogen response where the rhizobial density was less than 50 rhizobia g^{-1} soil was 375 %. Where the rhizobial density was greater than 50 rhizobia g^{-1} soil the mean response was 21.5 %.

A hyperbolic model, y=596.341/(1+MPN), where y=increase in shoot N due to inoculation expressed as a percent of the shoot N of uninoculated plants, best described the relationship between rhizobial density and inoculation response (Table 4.5). The residual mean square for this model was lower than that of linear, quadratic, and power models. Models where the y-intercept of the hyperbolic model was replaced with functions of soil N availability and symbiotic N demand had even lower residual mean square values (Table 4.5).

R. pseudoacacia Effectiveness Tests and Characterization of _R. pseudoacacia_ Rhizobial Isolates

All of the indigenous nodule isolates from *R. pseudoacacia* grown in Pane soil produced an acid reaction on bromothymol blue. They were distinctly different from isolates from *R. pseudoacacia* grown in Makawao soils which produced a neutral reaction. Of 23 rhizobial isolates from *R. pseudoacacia* in Pane soil, 18 nodulated both *Trifolium repens* and *Vicia* sp., and five nodulated *T. repens* but not *Vicia* sp. *Medicago sativa* did not form nodules with any of the strains. Five of the strains that nodulated *T. repens* were effective as evaluated by visual

observation of greener foliage and increased growth relative to uninoculated and ineffectively nodulated plants. Fifteen of the strains that nodulated *Vicia* sp. formed effective symbioses as demonstrated by pink to red nodule color in cross section. Of 20 *R. pseudoacacia* strains isolated from Makawao soil, 16 nodulated *L. diversifolia* effectively in tubes; 1 strain nodulated *T. repens* ineffectively. The effectiveness test revealed that the strains that nodulated *R. pseudoacacia* in Pane soil were ineffective while those from Makawao soil were at least moderately effective (Table 4.7).

DISCUSSION

The primary goal of our experiment was to determine the relationship between density of rhizobia in soil and response to inoculation of six tree legumes. The data and models generated from these experiments indicate a precipitous decline in response to inoculation as rhizobial density increases from 0 to 50 rhizobia g^{-1} soil. The relationship is very similar to those reported by Thies et al. (1991b) and Singleton et al. (1991a) for field-grown grain and forage legumes.

As in these experiments, Thies et al. (1991b), found that a hyperbolic model best described the relationship between rhizobial density and increase in economic yield due to inoculation. Measures of soil N availability and

symbiotic N demand improved the fit of their predictive models, as they did with the model developed from our data. Our data therefore suggest that the relationship between rhizobial density and response to inoculation is fundamentally the same with trees compared with grain and forage legumes and that mineral N has a similar effect on woody and non-woody legumes.

Species differences in MPN results and response to inoculation are consistent with what is known about the effectiveness groups of the trees used in this study. The data support the general view (Dommergues, 1987; Dreyfus and Dommergues, 1981; Peoples et al., 1989) that species nodulating with *Rhizobium* respond more often to inoculation than species nodulating with *Bradyrhizobium*. The data also support the separation of *Sesbania*, *Leucaena*, and *Robinia* into distinct effectiveness groups (Chapter 3) since both the density of indigenous rhizobia and magnitude of response to inoculation were species dependent. Effective nodulation of *R. pseudoacacia* with rhizobia capable of effective nodulation with *L. diversifolia* is consistent with observations of a moderately effective symbiosis between *R. pseudoacacia* and TAL 1145 (Chapter 3). The response of *R. pseudoacacia* to inoculation in the presence of effective strains does not support the conclusion of Singleton and Tavares (1986) that no response will occur in the presence of a large

rhizobial population as long as some strains are effective.

The inoculation response of *A. mearnsii* in Pane soil despite a rhizobial population of over 10 000 rhizobia g^{-1} soil supports the finding in Chapter 3 that *A. mearnsii* is promiscuous for nodulation but specific for effectiveness within the cowpea cross-inoculation group. The lower MPN estimates of *A. mangium* relative to *A. auriculiformis*, *A. mearnsii*, and *M. atropurpureum* in two soils support the finding in Chapter 3 that *A. mangium* is specific for infectiveness relative to these species.

The fact that *A. mangium* in Waiakoa soil accumulated substantially less N in uninoculated and inoculated pots relative to uninoculated *S. grandiflora* plants suggests that something other than rhizobia may have been limiting growth. The response of *A. mangium* to mineral N in this soil suggests that starter N may have been required.

Based on the tendency of MPN estimates of rhizobia in pure culture to be less than plate counts of the same cultures, a conservative recommendation would be to inoculate trees where MPN populations are less than 1000 rhizobia g^{-1} soil. If this recommendation is compared with actual results, inoculation would have been recommended for 14 of 24 tree-soil combinations, 10 of which responded to inoculation, but incorrectly recommending four species-soil combinations remain uninoculated where significant

inoculation responses in fact occurred: *R. pseudoacacia* in Pane, Makawao, and Waiakoa soils, and *A. mearnsii* in Pane soil. When effectiveness groups are taken into consideration, *R. pseudoacacia* can be expected to respond to inoculation in all soils because no legumes within its effectiveness group (in this case only *R. pseudoacacia* and perhaps other species in the genus *Robinia*) are present at any of the sites. In the same way *A. mearnsii* can be expected to respond to inoculation in Pane soil. With Makawao soil, although no *A. mearnsii* trees were present at the collection site, they do grow naturally in the general vicinity, which may explain the lack of response to inoculation in soil from this site.

In conclusion, leguminous trees respond to inoculation where rhizobial densities are low. As with grain and forage legumes, the magnitude of response to inoculation in tree legumes is inversely related to the density of rhizobia in the soil, with the greatest responses where rhizobial densities are less than 50 rhizobia g^{-1} soil. These experiments reveal that some tree legumes respond to inoculation despite the presence of high population densities of infective rhizobia in the soil, notably *R. pseudoacacia* and *A. mearnsii*. The reason appears to be that these species are specific for effectiveness but promiscuous for nodulation relative to indigenous rhizobia.

Table 4.1

Soils used in inoculation experiments and MPN assays.

Soil Series[a]	Soil Classification[b]	Maui Net Site	Mean Annual Rainfall[c] mm year^{-1}	Mineralized N g pot^{-1}[d]	%NDF[e]	pH[f]	Legumes Present at Site
Pane	Andisol	Pasture Kekoa	1060	5.35	79.7	6.2	*Desmodium* sp., *Medicago* sp., *Trifolium repens*, *Vicia sativa*
Makawao	Humoxic Tropohumult	Haleakala Station	1800	2.32	75.7	5.4	*Desmodium* sp., *Trifolium repens*, *Leucaena leucocephala*
Keahua	Aridic Haplustoll	Pasture Waiakoa	380	2.21	86.8	6.2	*Crotalaria* spp., *Prosopis* sp.
Waiakoa	Aridic Haplustoll	Hashimoto farm	322	0.94	92.2	6.3	*L. leucocephala*, *Crotalaria* spp., *Indigofera* sp.

[a] from Soil Conservation Service, 1972.
[b] from Dr. H. Ikawa, personal communication.
[c] from Department of Land and Natural Resources, 1982.
[d] calculated as g N/g of soil/week * 9 weeks * g soil/pot.
[e] % Nitrogen derived from fixation for *Sesbaina grandiflora*.
[f] 1:1 with water.

Table 4.2

Strains used in inoculation experiments and MPN assays.

Species	TAL No.	Original Host	Other Names
Acacia auriculiformis	* 569	*Desmodium uncinatum*	MAR 472
	1388	*Acacia mearnsii*	-
	*1446	*Acacia auriculiformis*	-
Acacia mangium	569	*Desmodium uncinatum*	MAR 472
	1388	*Acacia mearnsii*	-
	*1867	*Acacia mangium*	LB 5
Acacia mearnsii	132	*Acacia mearnsii*	-
	940	*Acacia mearnsii*	NUM 777
	* 941	*Acacia mearnsii*	NUM 778
Leucaena diversifolia	583	*Leucaena leucocephala*	NGR 8
	*1145	*Leucaena leucocephala*	CIAT 1967
	1887	*Leucaena leucocephala*	MS 111
Robinia pseudoacacia	183	*Robinia pseudoacacia*	NIT 137A4
	*1889	*Robinia pseudoacacia*	USDA 3436
	1907	*Robinia pseudoacacia*	USDA 3112
Sesbania grandiflora	1113	*Sesbania sp.*	IC 70
	1114	*Sesbania sp.*	IC 71
	*1119	*Sesbania sp.*	IC91
Macroptilium atropurpureum	169	*Vigna unguiculata*	NIT 176A22
	* 209	*Vigna radiata*	-
	1000	*Arachis hypogaea*	-

* = strain used for comparison of pure culture MPN estimates with plate counts.

Table 4.3. Rhizobial densities of six tree legumes and *Macroptilium atropurpureum* in four tropical soils and associated comparisons of pure culture plate counts with pure culture MPN estimates.

Species	Growth System	Soil				Pure Rhizobial Culture	
		Pane	Makawao	Keahua	Waiakoa	Pane & Makawao soils	Keahua & Waiakoa soils
		-rhizobia g^{-1} soil-				-Plate Count:MPN Ratio-	
Acacia auriculiformis	Tube	> 2510	> 26300	8.6	366	5.0	5.2
Acacia mangium	Tube	21.9	> 35600	41.3	6.8	17.9	5.0
Acacia mearnsii	Tube	49400	>227000	135	320	0.6	3.0
Leucaena diversifolia	Tube	< 0.2	>338000	6.1	9670	1.0	3.0
Robinia pseudoacacia	Pouch	21600	> 21600	39.5	6480	2.0	15.4
Sesbania grandiflora	Pouch	< 0.2	< 0.2	1.0	< 1.1	6.5	i.f.
Macroptilium atropurpureum	Pouch	18400	10500	6.5	27.2	i.f.[c]	14.9

[a] i.f. = inoculant failure: colonies formed on plates but plants did not nodulate.

Table 4.4

Shoot dry weight response to mineral N of six tree legumes.

Species	Soil	+N	Inoculated	Uninoculated
			--g pot^{-1}--	
Acacia mearnsii	Pane	24.5 a	9.1 b	6.5 b
Sesbania grandiflora	Pane	23.5 a	17.5 b	6.7 c
Leucaena diversifolia	Makawao	25.2 a	12.1 b	11.0 b
Acacia auriculiformis	Keahua	14.3 a	5.4 b	5.7 b
Robinia pseudoacacia	Keahua	20.4 a	8.8 b	2.4 c
Acacia mangium	Waiakoa	14.5 a	1.0 b	1.2 b
Sesbania grandiflora	Waiakoa	40.2 a	21.9 b	3.6 c

For each species-soil combination, means followed by the same letter are not significantly different by Tukey's HSD.

Table 4.5

Models of shoot N response to inoculation using measures of rhizobial density, mineralized N, and BNF potential.

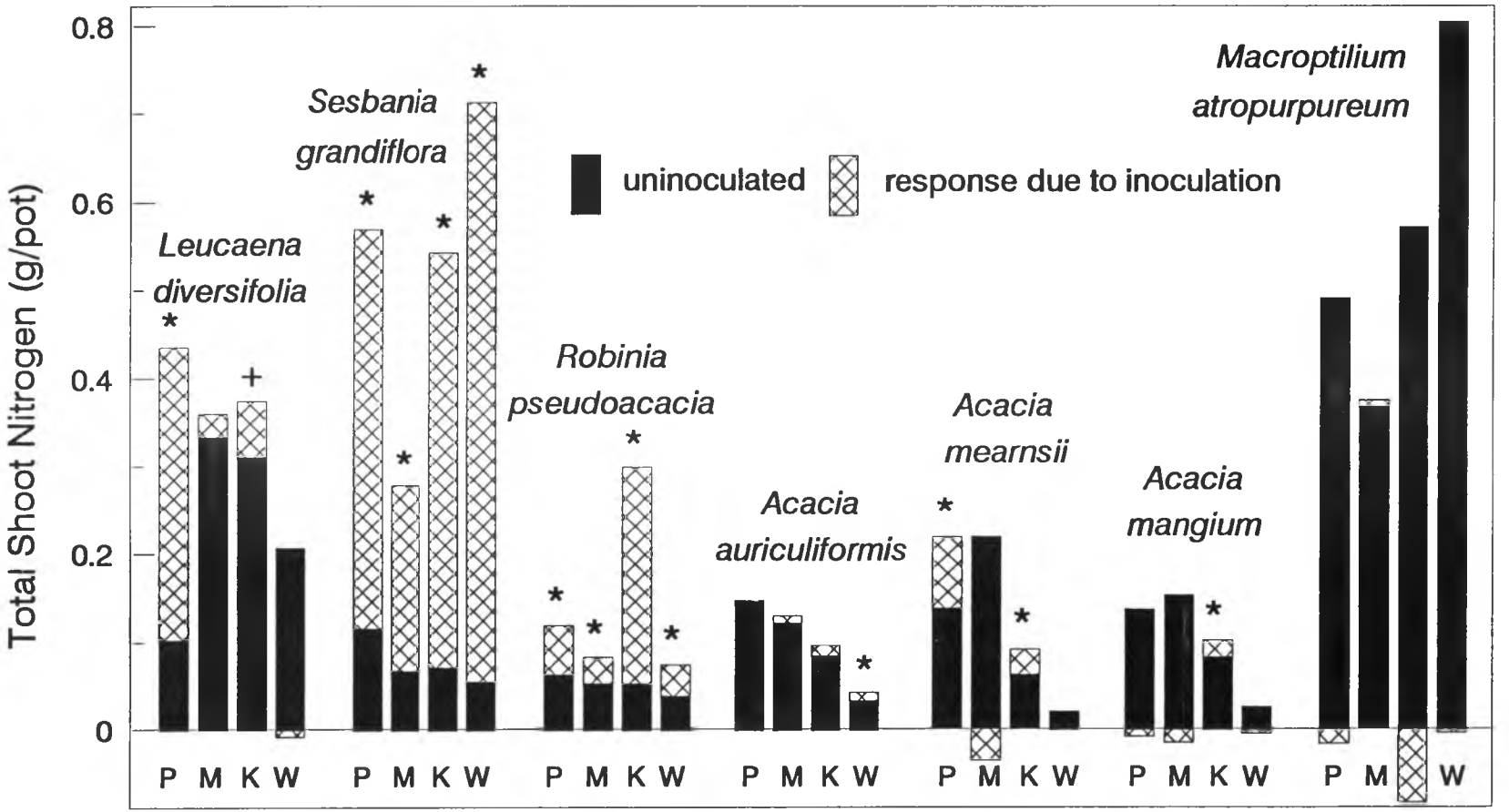
Model	Coefficients a	b	c	Residual Mean Square
Models with rhizobial density alone				
$a+bx$	191.0	-0.001		77550
ax^{-b}	550.3	20.84		54137
$a+b(\log x)+c(\log x)^2$	525.8	-129.7	7.368	41861
a/x	596.3			38467
Models with rhizobial density and mineralized N pot^{-1}				
$(a+bm)/x$	1105	-149.1		24883
$(ae^{-bm})/x$	1461	0.322		22184
$(am^{-b})/x$	1106	0.755		19630
$(a+b/m)/x$	155.4	947.9		19360
Models with rhizobial density and %NDF				
$(a+b/n)/x$	5521	-408196		18339
$(a+bn)/x$	-4370	59.63		17248
$(ae^{bn})/x$	0.143	0.098		15455

$x = 1 + $MPN estimate; y = shoot nitrogen response to inoculation expressed as a % of the uninoculated treatment; a = y-intercept; m = N mineralized pot^{-1}, n = %NDF of *S. grandiflora*.

Table 4.6. Effectiveness of indigenous vs. inoculant rhizobiai strains on *Robinia pseudoacacia* in growth pouches.

Pane soil	Treatment	Shoot dry weight g pouch-1
	inoculant strains	0.356 a*
	indigenous strains	0.071 b
	uninoculated control	0.063 b
Makawao soil		
	inoculant strains	0.353 a
	indigenous strains	0.275 a
	uninoculated control	0.064 b

For each soil, means followed by the same letter are not significantly different ($P < 0.05$) by Tukey's HSD



*, +: significant increase due to inoculation ($P < 0.05$ and $P < 0.10$ respectively)
soils: P = Pane, M = Makawao, K = Keahua, W = Waiakoa

Figure 4.1. Shoot N of six tree legumes and *Macroptilium atropurpureum* grown in four soils with and without inoculation.

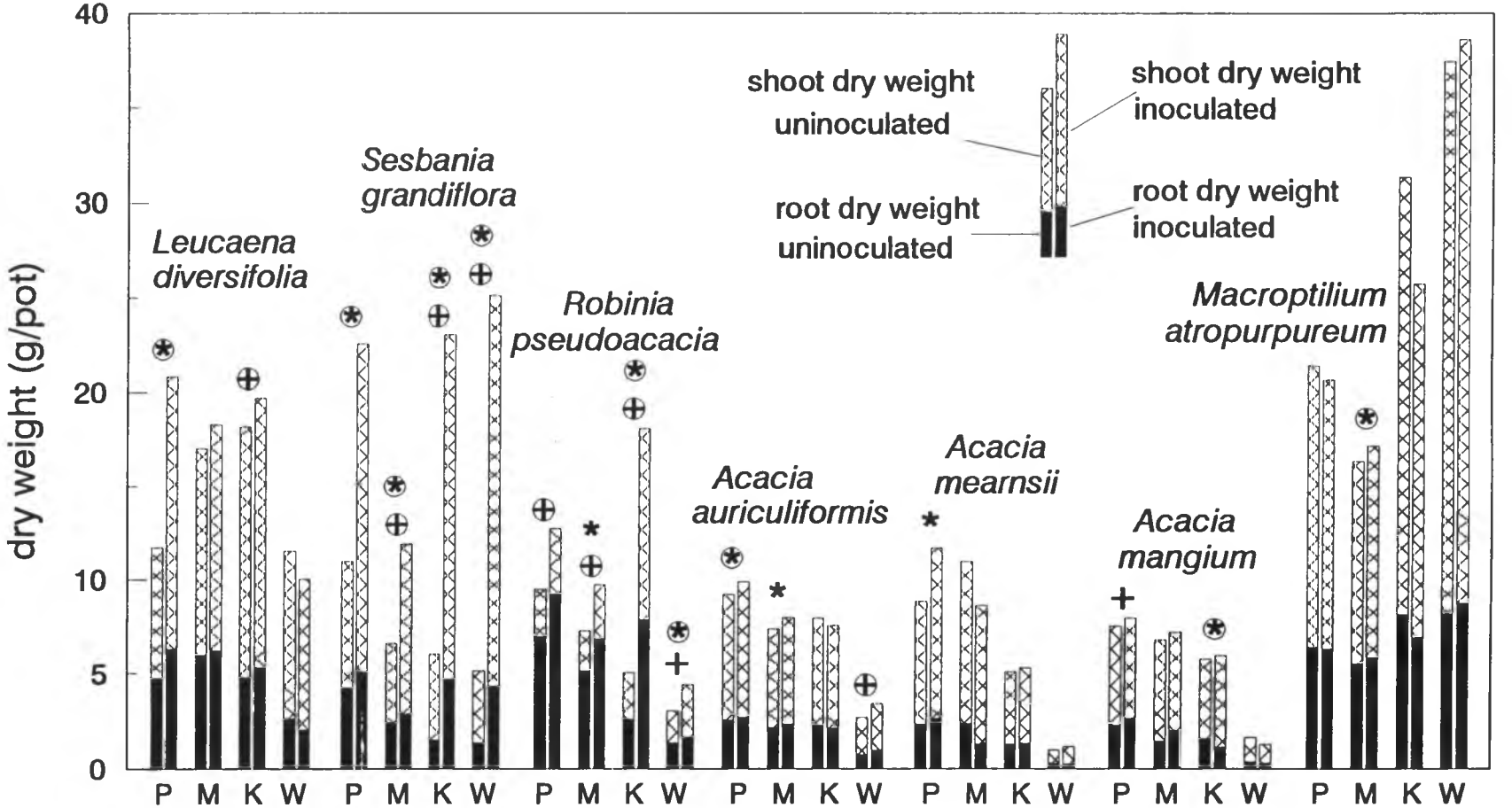
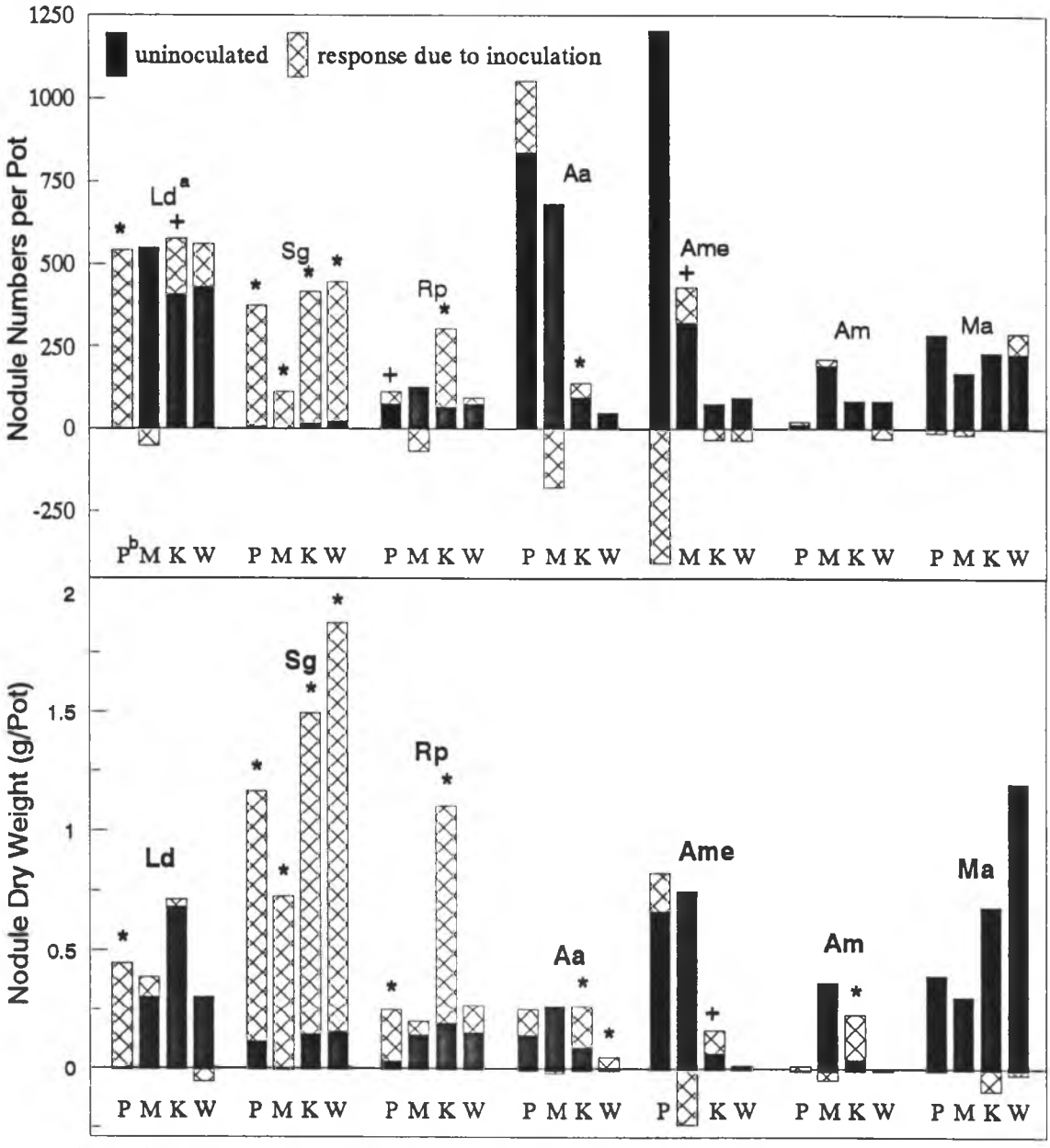


Figure 4.2. Shoot and root dry weight of six tree legumes and *Macroptilium atropurpureum* grown in four soils with and without inoculation.



*, + = increase due to inoculation at $P < 0.05$ and $P < 0.10$, respectively

[a] species: Aa = A. auriculiformis, Am = A. mangium, Ame = A. mearnsii, Ld = L. diversifolia
Rp = R. pseudoacacia, Sg = S. grandiflora, Ma = M. atropurpureum

[b] soils: P = Pane, M = Makawao, K = Keahua, W = Waiakoa

Figure 4.3. Nodule weight and numbers of six tree legumes and *Macroptilium atropurpureum* grown in four soils with and without inoculation.

Chapter 5

Thesis Conclusion

Despite the passage of over a century since BNF in trees was first studied (Allen and Allen, 1981), knowledge of rhizobial relationships among tree species remains far behind that of grain and forage legumes. This thesis has added to the body of knowledge by taking techniques and approaches developed with grain and forage legumes and applying them to trees. Specifically, rhizobial effectiveness groups were determined for a group of important tree legumes and this knowledge used in conjunction with measures of the density of indigenous rhizobial populations to determine the relationship between rhizobial density and response to inoculation.

Some of the contributions from this thesis are demonstration that reasonably accurate MPN estimates can be obtained with tree legumes, extension of the effectiveness group of *Leucaena* to include *Calliandra calothyrsus*, discovery that *Robinia pseudoacacia* and *Acacia mearnsii* are promiscuous for nodulation but specific for effectiveness, and confirmation that *Acacia mangium* is specific for both nodulation and effectiveness. Of practical importance, the data indicate that the approach developed for predicting yield response with grain and forage legumes can also be used with trees.

As a general conclusion, trees appear fundamentally similar to grain and forage legumes with respect to their rhizobial relations. As with grain and forage legumes, one rhizobial cell appears sufficient to cause nodule formation, a basic assumption of the MPN assay. Like other legumes, trees that nodulate effectively with *Rhizobium* fall into rather distinct effectiveness groups and species that nodulate effectively with *Bradyrhizobium* exhibit a range of specificity for both nodulation and effectiveness, without sharply delineated effectiveness groups. The minimal rhizobial density required for maximal BNF appears to be the same for trees and other legumes, in the order of 50 to 100 rhizobia g^{-1} soil as determined by the MPN assay. As with other legumes, mineral N attenuates the response to inoculation as indicated by the improved fit of models that included an index of available mineral N.

Several aspects of tree-rhizobia relationships touched on in this thesis warrant further investigation. In Chapter 2, although fairly accurate MPN estimates were obtained with 11 of 14 species, other growth systems need to be evaluated for the remaining species. Open tubes and Leonard jars might be worth consideration.

There has been very little published information pertaining to strain selection for tree legumes. Evidence of rhizobial specificity in tree legumes (Chapter 3),

especially for homologous strains, emphasizes the need to evaluate strains likely to have evolved in symbiosis with the species in question.

The response to inoculation of *R. pseudoacacia* and *A. mearnsii* in soils where rhizobial density was high emphasizes the need to develop tests for effectiveness to identify situations where species are likely to respond to inoculation despite high rhizobial densities. In this regard, an effectiveness test was conducted in association with the experiments presented in Chapter 4, but for several reasons it failed to identify tree-soil combinations where responses to inoculation occurred despite high rhizobial densities (Appendix C).

Uninoculated and inoculated treatments of *A. mangium* grew very poorly in Waiakoa soil despite a dramatic response to mineral N (Table 4.4). The fact that Waiakoa soil had the lowest available soil N as measured by the N mineralization test, and that inoculation increased yield of other tree species suggest that *A. mangium* has a higher requirement for starter N than species such as *S. grandiflora*. This and evidence that fast-growing species differ in their ability to fix N_2 in the presence of different levels of mineral N (George and Singleton, 1989) suggests that further work on relationships between N_2 fixation and mineral N might be fruitful, especially for species grown in association with food crops.

Appendix A

Generic determination of root nodule bacteria used in this thesis[a].

Strain		Genus[b]	BTB	XGal	Growth[c] on:	
Tal No.	Original host		reaction[c]	Reaction[c]	Sucrose	Lactose
1457	*Acacia albida*	*Bradyrhizobium*	alkaline	-		
1446	*Acacia auriculiformis*	*Bradyrhizobium*	alkaline	-		
1449	*Acacia auriculiformis*	*Bradyrhizobium*	alkaline	-		
1450	*Acacia auriculiformis*	*Bradyrhizobium*	alkaline	-		
1521	*Acacia auriculiformis*	*Bradyrhizobium*	alkaline	-		
1522	*Acacia auriculiformis*	*Bradyrhizobium*	alkaline	-		
881	*Acacia koa*	*Bradyrhizobium*	alkaline	-		
1867	*Acacia mangium*	*Bradyrhizobium*	alkaline	-		
1869	*Acacia mangium*	*Rhizobium*	acid	+		
63	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
111	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
112	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
126	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
132	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
133	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
134	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
179	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
180	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
940	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
941	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
942	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
1384	*Acacia mearnsii*	*Bradyrhizobium*	neutral	-		
1385	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
1386	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
1387	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
1388	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
1389	*Acacia mearnsii*	*Bradyrhizobium*	alkaline	-		
1852	*Albizia caribaea*	*Bradyrhizobium*	alkaline	-		
363	*Albizia lebbeck*	*Bradyrhizobium*		-	-	-
1536	*Albizia lebbeck*	*Bradyrhizobium*	alkaline	-		
1597	*Albizia lebbeck*	*Bradyrhizobium*	alkaline	-	-	-
1122	*Albizia stipulata*	*Bradyrhizobium*	alkaline	-		
1000	*Arachis hypogaea*	*Bradyrhizobium*	alkaline	-		
1371	*Arachis hypogaea*	*Bradyrhizobium*	alkaline	-		
33	*Calliandra calothyrsus*	*Rhizobium*		+		
1801	*Calliandra calothyrsus*	*Rhizobium*	acid	+		
1455	*Calliandra surinamensis*	*Rhizobium*	acid	+	+	+
651	*Calopogonium mucunoides*	*Bradyrhizobium*	alkaline	-		
201	*Canavalia ensiformis*	*Bradyrhizobium*	alkaline	-		
1380	*Crotalaria paulina*	*Bradyrhizobium*	alkaline	-		
850	*Crotalaria sp.*	*Bradyrhizobium*	alkaline	-		
569	*Desmodium uncinatum*	*Bradyrhizobium*	alkaline	-		
47	*Enterolobium cyclocarpum*	*Bradyrhizobium*	alkaline	-		
60	*Enterolobium cyclocarpum*	*Bradyrhizobium*	alkaline	-		
1530	*Enterolobium cyclocarpum*	*Bradyrhizobium*	alkaline	-		
69	*Erythrina indica*	*Bradyrhizobium*	alkaline	-		
749	*Erythrina indica*	*Bradyrhizobium*	alkaline	-		
1883	*Flemingia macrophylla*	*Bradyrhizobium*	alkaline	-		
1788	*Gliricidia maculata*	*Rhizobium*		+		
7	*Gliricidia sepium*	*Rhizobium*	acid	+		
1770	*Gliricidia sepium*	*Rhizobium*	acid	+		
1806	*Gliricidia sepium*	*Rhizobium*	acid	+	+	+
102	*Glycine max*	*Bradyrhizobium*	alkaline	-		
1908	*Glycine max*	*Bradyrhizobium*	alkaline	-		
82	*Leucaena leucocephala*	*Rhizobium*	acid		+	+
582	*Leucaena leucocephala*	*Rhizobium*		+	+	+
583	*Leucaena leucocephala*	*Rhizobium*	acid	+		
1145	*Leucaena leucocephala*	*Rhizobium*	acid	+	+	+
1887	*Leucaena leucocephala*	*Rhizobium*	acid	-	+	+

Appendix A (Continued)

Generic determination of root nodule bacteria used in this thesis

Strain		Genus[a]	BTB[a]	X-Gal[a]	Growth[a] on:	
Tal No.	Original host		reaction	Reaction	Sucrose	Lactose
309	*Macrotyloma africanum*	*Bradyrhizobium*	alkaline	-		
45	*Paraserianthes falcataria*	*Bradyrhizobium*	alkaline	-		
644	*Phaseolus acutifolius*	*Bradyrhizobium*	alkaline	-		
22	*Phaseolus lunatus*	*Bradyrhizobium*	alkaline	-		
183	*Robinia pseudoacacia*	*Rhizobium*	neutral	-	+	+
1889	*Robinia pseudoacacia*	*Rhizobium*	acid	-	+	-
1907	*Robinia pseudoacacia*	*Rhizobium*	acid	+		
833	*Samanea saman*	*Bradyrhizobium*	alkaline	-		
1280	*Samanea saman*	*Bradyrhizobium*	alkaline	-		
1779	*Sesbania grandiflora*	*Rhizobium*	acid	-	+	+
1042	*Sesbania longifolia*	*Rhizobium*	acid	+		
1886	*Sesbania longifolia*	*Rhizobium*	acid	+		
674	*Sesbania rostrata*	*Rhizobium*	acid	+		
1829	*Sesbania rostrata*	*Azorhizobium*[f]	alkaline	-	-	-
1113	*Sesbania sp.*	*Rhizobium*	acid	+		
1114	*Sesbania sp.*	*Rhizobium*	acid	+		
1119	*Sesbania sp.*	*Rhizobium*	acid	+		
1137	*Sesbania sp.*	*Rhizobium*	acid	+		
658	*Stylosanthes sp.*	*Bradyrhizobium*		-	-	-
795	*Tephrosia glauca*	*Bradyrhizobium*	alkaline	-		
209	*Vigna radiata*	*Bradyrhizobium*	alkaline	-		
169	*Vigna unguiculata*	*Bradyrhizobium*	alkaline	-		
173	*Vigna unguiculata*	*Bradyrhizobium*	alkaline	-	-	-

[a] = tests conducted by H.H. Keyser.

[b] = Rhizobium if XGal reaction positive or strain grows on either sucrose or lactose; = Bradyrhizobium if BTB reaction is alkaline and XGal test is negative.

[c] = reaction on bromothymol blue agar determined between 3 and 7 days after plating.

[d] = IPTG -XGal test for detection of ß-galactosidase activity *α*-complementation (Sambrook et al., 1989). Determination made 10 days after plating.

[e] = growth on dissacharides determined after 7 days.

[f] = a characterized strain of *Azorhizobium*.

Appendix B

Data for individual MPN assays.

Species	Strain TAL #	Growth System	Dilution Factor	MPN Code	MPN Estimate	Factor for 95% C.I. of MPN	Plate Count	95% Confidence Interval for Plate Count	PC within C.I. of MPN	PC:MPN Ratio	Nodulated Uninoculated Controls	# of Weeks to Scoring
Acacia	569	pouch	3.9	43100000	4.22E+06	2.64	3.15E+09	2.510E+09 - 3.79E+09	N	746.4	0/8	7
auriculiformis	1446	pouch	3.9	43111000	2.61E+06	2.64	5.84E+09	5.100E+09 - 6.58E+09	N	2237.5	0/8	7
	1446	pouch	10.0	2100000	9.25E+04	3.80	1.96E+09	1.600E+09 - 2.32E+09	N	21189.2	1/4	5
	651	pouch	4.3	00000000	<1.73E+06	2.74	7.91E+08	6.90E+08 - 8.92E+08	N	>457.2*	0/8	5
	1446	tube	3.9	4434430	1.16E+09	2.64	5.84E+09	5.10E+09 - 6.58E+09	N	5.0	0/8	7
	569	tube	3.9	443412000	6.10E+08	2.64	3.15E+09	2.51E+09 - 3.79E+09	N	5.2	0/8	7
	651	tube	4.3	40000000	1.18E+07	2.74	7.91E+08	6.90E+08 - 8.92E+08	N	67.0	0/8	5
Acacia	1867	pouch	3.9	44321100	1.27E+07	2.64	6.57E+09	5.92E+09 - 7.22E+09	N	517.3	0/8	7
mangium	1867	pouch	4.0	441000	7.76E+05	2.68	5.90E+08	4.87E+08 - 6.93E+08	N	760.3	0/8	4
	1867	pouch	3.9	200000000	4.20E+05	2.64	2.45E+09	2.03E+09 - 2.87E+09	N	5833.3	0/8	7
	1867	pouch	4.1	000000	<1.30E+06	2.68	5.90E+08	4.87E+08 - 6.93E+08	N	>453.8*	0/8	5
	1867	tube	3.9	44433100	4.88E+08	2.64	2.45E+09	2.03E+09 - 2.87E+09	N	5.0	0/8	7
	1867	tube	3.9	4444332211	3.68E+08	2.64	6.57E+09	5.92E+09 - 7.22E+09	N	17.9	0/8	7
	1867	tube	4.1	433000	5.20E+07	2.68	1.04E+09	8.91E+08 - 1.19E+09	N	20.0	0/8	5
	1867	tube	4.1	441000	4.96E+07	2.68	1.04E+09	8.91E+08 - 1.19E+09	N	21.0	0/8	5
Acacia mearnsii	941	pouch	3.9	44444410	6.05E+08	2.64	3.34E+09	2.98E+09 - 3.70E+09	N	5.5	0/8	7
	941	pouch	3.9	43110000	2.03E+07	2.64	8.11E+08	6.64E+08 - 9.58E+08	N	40.0	0/8	7
	1388	pouch	4.0	430100	4.75E+05	2.68	3.13E+08	2.54E+08 - 3.72E+08	N	658.9	0/4	4
	940	pouch	4.1	0000000	<1.30E+06	2.68	1.32E+08	1.17E+08 - 1.47E+08	N	>101.5*	0/8	5
	941	tube	3.9	4444434210	5.84E+09	2.64	3.34E+09	2.98E+09 - 3.70E+09	Y	0.6	0/8	7
	941	tube	3.9	34400000	2.70E+08	2.64	8.11E+08	6.64E+08 - 9.58E+08	Y	3.0	0/8	7
	940	tube	4.1	43220000	6.07E+07	2.68	1.32E+08	1.17E+08 - 1.47E+08	Y	2.2	0/8	5
	940	tube	4.1	4321100	6.01E+07	2.68	1.32E+08	1.17E+08 - 1.47E+08	Y	2.2	0/8	5
Albizia lebbeck	1536	pouch	10.0	3412000	7.97E+06	3.80	9.47E+08	7.78E+08 - 1.12E+09	N	118.8	0/7	5
Albizia saman	833	pouch	10.0	1000000	2.55E+04	3.80	3.27E+08	2.51E+08 - 4.03E+08	N	12823.5	0/5	5
Calliandra calothyrsus	1455	pouch	10.0	4444100	3.59E+08	3.80	7.60E+08	6.57E+08 - 8.63E+08	Y	2.1	0/2	5
Flemingia macrophylla	1883	pouch	10.0	42110000	1.26E+06	3.80	1.09E+09	8.10E+08 - 1.37E+09	N	865.1	0/4	6
Gliricidia	1145	pouch	10.0	44400000	2.31E+07	3.80	3.00E+08	1.65E+08 - 4.35E+08	N	13.0	0/5	6
sepium	1806	pouch	10.0	4444300	1.12E+09	3.80	1.25E+09	1.06E+09 - 1.44E+09	Y	1.1	1/6	5

Appendix B (Continued)

Data for individual MPN assays.

Species	Strain TAL #	Growth System	Dilution Factor	MPN Code	MPN Estimate	Factor for 95% C.I. of MPN	Plate Count	95% Confidence Interval for Plate Count	PC within C.I. of MPN	PC:MPN Ratio	Nodulated Uninoculated Controls	# of Weeks to Scoring
Leucaena	1145	pouch	3.9	4444400000	4.01E+09	2.64	3.82E+09	3.26E+09 - 4.38E+09	Y	1.0	0/8	7
diversifolia	1145	pouch	4.2	44311000	1.97E+08	2.72	3.36E+08	2.96E+08 - 3.76E+08	Y	1.7	0/8	5
	1145	tube	3.9	4444443200	7.56E+09	2.64	7.55E+09	5.98E+09 - 9.12E+09	Y	1.0	0/8	7
	1145	tube	4.2	444441000	1.06E+09	2.72	3.36E+08	2.96E+08 - 3.76E+08	N	0.3	0/8	5
	1145	tube	3.9	444301000	9.06E+08	2.64	3.82E+09	3.26E+09 - 4.38E+09	N	4.2	0/8	7
	1145	tube	4.2	444430000	4.96E+08	2.72	3.36E+08	2.96E+08 - 3.76E+08	Y	0.7	0/8	5
Leucaena	1145	pouch	10.0	43320000	4.09E+06	3.8	2.71E+08	2.15E+08 - 3.27E+08	N	66.3	0/7	5
leucocephala	1145	pouch	10.0	4433100	3.40E+07	3.8	1.22E+09	1.15E+09 - 1.29E+09	N	35.9	1/7	5
	1145	pouch	4.0	4444410000	5.01E+07	2.68	3.67E+07	3.07E+07 - 4.27E+07	Y	0.7	0/4	6
Paraserianthes	1536	pouch	4.2	11000000	7.35E+05	2.72	6.50E+08	5.04E+08 - 7.96E+08	N	884.4	0/8	5
falcataria	45	pouch	4.0	4422000000	1.75E+06	2.68	3.73E+08	2.26E+08 - 5.20E+08	N	213.1	0/4	6
	1536	tube	4.2	44420000	3.49E+08	2.72	6.50E+08	5.04E+08 - 7.96E+08	Y	1.9	0/8	5
	1536	tube	4.2	44431000	6.54E+08	2.72	6.50E+08	5.04E+08 - 7.96E+08	Y	1.0	0/8	5
Robinia	1889	pouch	3.9	44442200	1.35E+09	2.64	2.72E+09	2.46E+09 - 2.98E+09	Y	2.0	0/8	7
pseudoacacia	1889	pouch	4.0	44442000	6.82E+08	2.68	9.33E+07	7.40E+07 - 1.13E+08	N	0.1	0/8	5
	1889	pouch	3.9	444000100	2.70E+08	2.64	4.17E+09	2.85E+09 - 5.49E+09	N	15.4	0/8	7
	1889	tube	3.9	4444443300	2.52E+09	2.64	2.72E+09	2.46E+09 - 2.98E+09	Y	1.1	0/8	7
	1889	tube	4.0	44440100	4.73E+08	2.68	9.33E+07	7.40E+07 - 1.13E+08	N	0.2	0/7	5
	1889	tube	3.9	44100000	9.73E+07	2.64	4.17E+09	2.85E+09 - 5.49E+09	N	42.9	0/8	7
Sesbania	1114	pouch	4.0	4322201	5.21E+07	2.68	4.58E+08	4.10E+08 - 5.06E+08	N	8.8	1/6	6
grandiflora	1119	pouch	3.9	4444000	4.49E+08	2.64	2.93E+09	2.53E+09 - 3.33E+09	N	6.5	0/8	7
	1114	pouch	4.2	4443000	4.96E+08	2.72	9.60E+08	7.74E+08 - 1.15E+09	Y	1.9	0/8	5
	1114	tube	4.0	11100000	2.60E+06	2.68	4.58E+08	4.10E+08 - 5.06E+08	N	176.2	0/8	6
	1114	tube	4.2	00010000	<3.58E+05	2.72	9.60E+08	7.74E+08 - 1.15E+09	N	>2681.6	0/8	5
Sesbania	1042	pouch	4.0	444444201	3.49E+08	2.68	6.13E+08	5.71E+08 - 6.55E+08	Y	1.8	0/3	7
sesban	674	pouch	10.0	4444200	6.14E+08	3.8	6.73E+08	5.67E+08 - 7.79E+08	Y	1.1	0/7	5
	1042	pouch	10.0	44443012	1.20E+09	3.8	1.03E+09	8.46E+08 - 1.21E+09	Y	0.9	1/6	4

* = not used for means in Table 2.4.

Appendix C

Results of effectiveness tests in pouches[a].

Species	Soil	No. reps	Plants/ pouch	Treatments: Steamed Soil (A)	Treatments: Soil (B)	Treatments: Soil + Rhizobia (C)	Two-way comparisons[b] -P-	
				Shoot dry weight (mg/plant)			B vs. C	A vs. B
A. auriculiformis	Pane	8	1	66	90	112	0.087	0.041
A. mangium	Pane	8	1	16	12	24	0.000	1.000
A. mearnsii	Pane	7	2	72	41	83	0.000	1.000
L. diversifolia	Pane	7	2	74	46	170	0.000	1.000
R. pseudoacacia	Pane	6	2	121	73	300	0.0025	1.000
S. grandiflora	Pane	7	1	117	59	485	0.001	1.000
A. auriculiformis	Keahua	6	2	104	97	160	0.007	1.000
A. mangium	Keahua	6	2	30	20	48	0.002	1.000
A. mearnsii	Keahua	6	2	43	28	95	0.0025	1.000
L. diversifolia	Keahua	6	2	45	62	147	0.001	0.170
R. pseudoacacia	Keahua	6	2	107	79	342	0.0055	1.000
S. grandiflora	Keahua	4	2	111	121	566	0.0025	0.343
A. auriculiformis	Waiakoa	6	1	44	48	67	0.0015	0.241
A. mangium	Waiakoa	6	1	10	12	24	0.0125	0.253
A. mearnsii	Waiakoa	6	2	29	37	113	0.000	0.064
L. diversifolia	Waiakoa	6	1	18	97	61	1.000	0.001
R. pseudoacacia	Waiakoa	6	1	41	42	72	0.0395	0.418
S. grandiflora	Waiakoa	6	1	43	48	384	0.001	0.291

[a] Pouches were prepared as for MPN assays (Chapter 2), each pouch received an inoculum consisting of 10 g (dry weight equivalent) soil from an uninoculated pot in the inoculation experiment (Chapter 4) of one of three treatments: soil steamed for 45 min. at 110°C, unsteamed soil, or unsteamed soil to which rhizobia were added (approximately 10^6 rhizobia pouch^{-1} of the same three-strain mixture used for inoculation in of the inoculation experiments). Plants were harvested after 6 weeks.

[b] = by one-tailed t test.

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